US006162461A

# United States Patent [19]

**Noteborn et al.**

[11] **Patent Number: 6,162,461**

[45] **Date of Patent: *Dec. 19, 2000**

[54] **CHICKEN ANEMIA VIRUS MUTANTS AND VACCINES AND USES BASED ON THE VIRAL PROTEINS VP1, VP2 AND VP3 OR SEQUENCES OF THAT VIRUS CODING THEREFOR**

[75] Inventors: **Matheus Hubertus Maria Noteborn**, Leiden; **Guus Koch**, Lelystad, both of Netherlands

[73] Assignee: **Leadd B.V.**, Leiden, Netherlands

[ * ] Notice: This patent is subject to a terminal disclaimer.

[21] Appl. No.: **08/482,161**

[22] Filed: **Jun. 7, 1995**

**Related U.S. Application Data**

[62] Continuation-in-part of application No. 08/454,121, filed as application No. PCT/NL94/00168, Jul. 19, 1994, which is a continuation-in-part of application No. 08/030,335, filed as application No. PCT/NL91/00165, Sep. 11, 1991, Pat. No. 5,491,073.

[30] **Foreign Application Priority Data**

Sep. 12, 1990 [NL] Netherlands .......................... 9002008
Jul. 20, 1993 [NL] Netherlands .......................... 9301272

[51] **Int. Cl.**[7] .......................... **A61K 38/16**; A61K 9/127; C12N 15/87; C07K 14/01

[52] **U.S. Cl.** ..................... **424/450**; 424/178.1; 424/93.2; 514/2; 514/44; 435/235.1; 435/455; 435/456; 435/459; 435/69.1; 530/350

[58] **Field of Search** ............................. 424/186.1, 204.1, 424/450, 178.1, 93.2; 514/2, 44, 12, 14; 530/387.9, 327, 350; 536/23.72; 435/5, 235.1, 455, 456, 459, 69.1

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(List continued on next page.)

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[57] **ABSTRACT**

The coding information for three putative chicken anemia virus proteins (VP1, VP2, VP3) was inserted into a baculovirus vector and expressed in insect cells. The immunogenic properties of the chicken anemia virus (CAV) proteins produced separately or together in insect-cell cultures were analyzed by inoculating them into chickens. Only lysates of insect cells which have synthesized equivalent amounts of all three recombinant CAV proteins or cells which synthesized mainly VP1 plus VP2 induced neutralizing antibodies directed against CAV in inoculated chickens. Progeny of those chickens were protected against clinical disease after CAV challenge. Inoculation of a mixture of lysates of cells that were separately infected with VP1-, VP2- and VP3-recombinant baculovirus did not induce significant levels of neutralizing antibody directed against CAV and their progeny were not protected against CAV challenge. Our results indicate that expression in the same cell of at least two CAV proteins, VP1 plus VP2, is required to obtain sufficient protection in chickens. Therefore, recombinant CAV proteins produced by baculovirus vectors can be used as a sub-unit vaccine against CAV infections.

**42 Claims, 15 Drawing Sheets**

```
 M  N  A  L  Q  E  D  T  P  P  G  P  S  T  V  F  R  P  P  T
ATGAACGCTCTCCAAGAAGATACTCCACCCGGACCATCAACGGTGTTCAGGCCACCAACA    545
 S  S  R  P  L  E  T  P  H  C  R  E  I  R  I  G  I  A  G  I
AGTTCACGGCCGTTGGAAACCCCTCACTGCAGAGAGATCCGGATTGGTATCGCTGGAATT    605
 T  I  T  L  S  L  C  G  C  A  N  A  R  A  P  T  L  R  S  A
ACAATCACTCTATCGCTGTGTGGCTGCGCGAATGCTCGCGCTCCCACGCTAAGATCTGCA    665
 T  A  D  N  S  E  S  T  G  F  K  N  V  P  D  L  R  T  D  Q
ACTGCGGACAATTCAGAAAGCACTGGTTTCAAGAATGTGCCGGACTTGAGGACCGATCAA    725
 P  K  P  P  S  K  K  R  S  C  D  P  S  E  Y  R  V  S  E  L
CCCAAGCCTCCCTCGAAGAAGCGATCCTGCGACCCCTCCGAGTACAGGGTAAGCGAGCTA    785
 K  E  S  L  I  T  T  T  P  S  R  P  R  T  A  K  R  R  I  R
AAAGAAAGCTTGATTACCACTACTCCCAGCCGACCCCGAACCGCAAAAAGGCGTATAAGA    845
 L  *
CTGTAA                                                          851
```

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```
 M  A  R  R  A  R  R  P  R  G  R  F  Y  S  F  R  R  G  R  W
ATGGCAAGACGAGCTCGCAGACCGAGAGGCCGATTTTACTCCTTCAGAAGAGGACGGTGG   912
 H  H  L  K  R  L  R  R  R  Y  K  F  R  H  R  R  R  Q  R  Y
CACCACCTCAAGCGACTTCGACGAAGATATAAATTTCGACATCGGAGGAGACAGCGGTAT   972
 R  R  R  A  F  R  K  A  F  H  N  P  R  P  G  T  Y  S  V  R
CGTAGACGAGCTTTTAGGAAGGCCTTTCACAACCCCCGCCCCGGTACGTATAGTGTGAGG  1032
 L  P  N  P  Q  S  T  M  T  I  R  F  Q  G  V  I  F  L  T  E
CTGCCGAACCCCCAATCTACTATGACTATCCGCTTCCAAGGGGTCATCTTTCTCACGGAA  1092
 G  L  I  L  P  K  N  S  T  A  G  G  Y  A  D  H  M  Y  G  A
GGACTCATTCTGCCTAAAAACAGCACAGCGGGGGGCTATGCAGACCACATGTACGGGGCG  1152
 R  V  A  K  I  S  V  N  L  K  E  F  L  L  A  S  M  N  L  T
AGAGTCGCCAAGATCTCTGTGAACCTGAAAGAGTTCCTGCTAGCCTCAATGAACCTGACA  1212
 Y  V  S  K  I  G  G  P  I  A  G  E  L  I  A  D  G  S  K  S
TACGTGAGCAAAATCGGAGGCCCCATCGCCGGTGAGTTGATTGCGGACGGGTCTAAATCA  1272
 Q  A  A  D  N  W  P  N  C  W  L  P  L  D  N  N  V  P  S  A
CAAGCCGCGGACAATTGGCCTAATTGCTGGCTGCCGCTAGATAATAACGTGCCCTCCGCT  1332
 T  P  S  A  W  W  R  W  A  L  M  M  M  Q  P  T  D  S  C  R
ACACCATCGGCATGGTGGAGATGGGCCTTAATGATGATGCAGCCCACGGACTCTTGCCGG  1392
 F  F  N  H  P  K  Q  M  T  L  Q  D  M  G  R  M  F  G  G  W
TTCTTTAATCACCCAAAGCAGATGACCCTGCAAGACATGGGTCGCATGTTTGGGGGCTGG  1452
 H  L  F  R  H  I  E  T  R  F  Q  L  L  A  T  K  N  E  G  S
CACCTGTTCCGACACATTGAAACCCGCTTTCAGCTCCTTGCCACTAAGAATGAGGGATCC  1512
 F  S  P  V  A  S  L  L  S  Q  G  E  Y  L  T  R  R  D  D  V
TTCAGCCCCGTGGCGAGTCTTCTCTCCCAGGGAGAGTACCTCACGCGTCGGGACGATGTT  1572
 K  Y  S  S  D  H  Q  N  R  W  Q  K  G  G  Q  P  M  T  G  G
AAGTACAGCAGCGATCACCAGAACCGGTGGCAAAAAGGCGGACAACCGATGACGGGGGGC  1632
 I  A  Y  A  T  G  K  M  R  P  D  E  Q  Q  Y  P  A  M  P  P
ATTGCTTATGCGACCGGGAAAATGAGACCCGACGAGCAACAGTACCCTGCTATGCCCCCA  1692
 D  P  P  I  I  T  A  T  T  A  Q  G  T  Q  V  R  C  M  N  S
GACCCCCCGATCATCACCGCTACTACAGCGCAAGGCACGCAAGTCCGCTGCATGAATAGC  1752
 T  Q  A  W  W  S  W  D  T  Y  M  S  F  A  T  L  T  A  L  G
ACGCAAGCTTGGTGGTCATGGGACACATATATGAGCTTTGCAACACTCACAGCACTCGGT  1812
 A  Q  W  S  F  P  P  G  Q  R  S  V  S  R  R  S  F  N  H  H
GCACAATGGTCTTTTCCTCCAGGGCAACGTTCAGTTTCTAGACGGTCCTTCAACCACCAC  1872
 K  A  R  G  A  G  D  P  K  G  Q  R  W  H  T  L  V  P  L  G
AAGGCGAGAGGAGCCGGGGACCCCAAGGGCCAGAGATGGCACACGCTGGTGCCGCTCGGC  1932
 T  E  T  I  T  D  S  Y  M  S  A  P  A  S  E  L  D  T  N  F
ACGGAGACCATCACCGACAGCTACATGTCAGCACCCGCATCAGAGCTGGACACTAATTTC  1992
 F  T  L  Y  V  A  Q  G  T  N  K  S  Q  Q  Y  K  F  G  T  A
TTTACGCTTTACGTAGCGCAAGGCACAAATAAGTCGCAACAGTACAAGTTCGGCACAGCT  2052
 T  Y  A  L  K  E  P  V  M  K  S  D  A  W  A  V  V  R  V  Q
ACATACGCGCTAAAGGAGCCGGTAATGAAGAGCGATGCATGGGCAGTGGTACGCGTCCAG  2112
 S  V  W  Q  L  G  N  R  Q  R  P  Y  P  W  D  V  N  W  A  N
TCGGTCTGGCAGCTGGGTAACAGGCAGAGGCCATACCCATGGGACGTCAACTGGGCGAAC  2172
 S  T  M  Y  W  G  T  Q  P  *
AGCACCATGTACTGGGGGACGCAGCCCTGA                                2201
```

FIG. 1

```
 M  H  G  N  G  G  Q  P  A  A  G  G  S  E  S  A  L  S  R  E
ATGCACGGGAACGGCGGACAACCGGCCGCTGGGGGCAGTGAATCGGCGCTTAGCCGAGAG    439
 G  Q  P  G  P  S  G  A  A  Q  G  Q  V  I  S  N  E  R  S  P
GGGCAACCTGGGCCCAGCGGAGCCGCGCAGGGGCAAGTAATTTCAAATGAACGCTCTCCA    499
 R  R  Y  S  T  R  T  I  N  G  V  Q  A  T  N  K  F  T  A  V
AGAAGATACTCCACCCGGACCATCAACGGTGTTCAGGCCACCAACAAGTTCACGGCCGTT    559
 G  N  P  S  L  Q  R  D  P  D  W  Y  R  W  N  Y  N  H  S  I
GGAAACCCCTCACTGCAGAGAGATCCGGATTGGTATCGCTGGAATTACAATCACTCTATC    619
 A  V  W  L  R  E  C  S  R  S  H  A  K  I  C  N  C  G  Q  F
GCTGTGTGGCTGCGCGAATGCTCGCGCTCCCACGCTAAGATCTGCAACTGCGGACAATTC    679
 R  K  H  W  F  Q  E  C  A  G  L  E  D  R  S  T  Q  A  S  L
AGAAAGCACTGGTTTCAAGAATGTGCCGGACTTGAGGACCGATCAACCCAAGCCTCCCTC    739
 E  E  A  I  L  R  P  L  R  V  Q  G  K  R  A  K  R  K  L  D
GAAGAAGCGATCCTGCGACCCCTCCGAGTACAGGGTAAGCGAGCTAAAAGAAAGCTTGAT    799
 Y  H  Y  S  Q  P  T  P  N  R  K  K  A  Y  K  T  V  R  W  Q
TACCACTACTCCCAGCCGACCCCGAACCGCAAAAAGGCGTATAAGACTGTAAGATGGCAA    859
 D  E  L  A  D  R  E  A  D  F  T  P  S  E  E  D  G  G  T  T
GACGAGCTCGCAGACCGAGAGGCCGATTTTACTCCTTCAGAAGAGGACGGTGGCACCACC    919
 S  S  D  F  D  E  D  I  N  F  D  I  G  G  D  S  G  I  V  D
TCAAGCGACTTCGACGAAGATATAAATTTCGACATCGGAGGAGACAGCGGTATCGTAGAC    979
 E  L  L  G  R  P  F  T  T  P  A  P  V  R  I  V  *
GAGCTTTTAGGAAGGCCTTTCACAACCCCCGCCCCGGTACGTATAGTGTGA            1030
```

FIG. 2

```
 M  N  A  L  Q  E  D  T  P  P  G  P  S  T  V  F  R  P  P  T
ATGAACGCTCTCCAAGAAGATACTCCACCCGGACCATCAACGGTGTTCAGGCCACCAACA   545
 S  S  R  P  L  E  T  P  H  C  R  E  I  R  I  G  I  A  G  I
AGTTCACGGCCGTTGGAAACCCCTCACTGCAGAGAGATCCGGATTGGTATCGCTGGAATT   605
 T  I  T  L  S  L  C  G  C  A  N  A  R  A  P  T  L  R  S  A
ACAATCACTCTATCGCTGTGTGGCTGCGCGAATGCTCGCGCTCCCACGCTAAGATCTGCA   665
 T  A  D  N  S  E  S  T  G  F  K  N  V  P  D  L  R  T  D  Q
ACTGCGGACAATTCAGAAAGCACTGGTTTCAAGAATGTGCCGGACTTGAGGACCGATCAA   725
 P  K  P  P  S  K  K  R  S  C  D  P  S  E  Y  R  V  S  E  L
CCCAAGCCTCCCTCGAAGAAGCGATCCTGCGACCCCTCCGAGTACAGGGTAAGCGAGCTA   785
 K  E  S  L  I  T  T  T  P  S  R  P  R  T  A  K  R  R  I  R
AAAGAAAGCTTGATTACCACTACTCCCAGCCGACCCCGAACCGCAAAAAGGCGTATAAGA   845
 L  *
CTGTAA                                                         851
```

FIG. 3

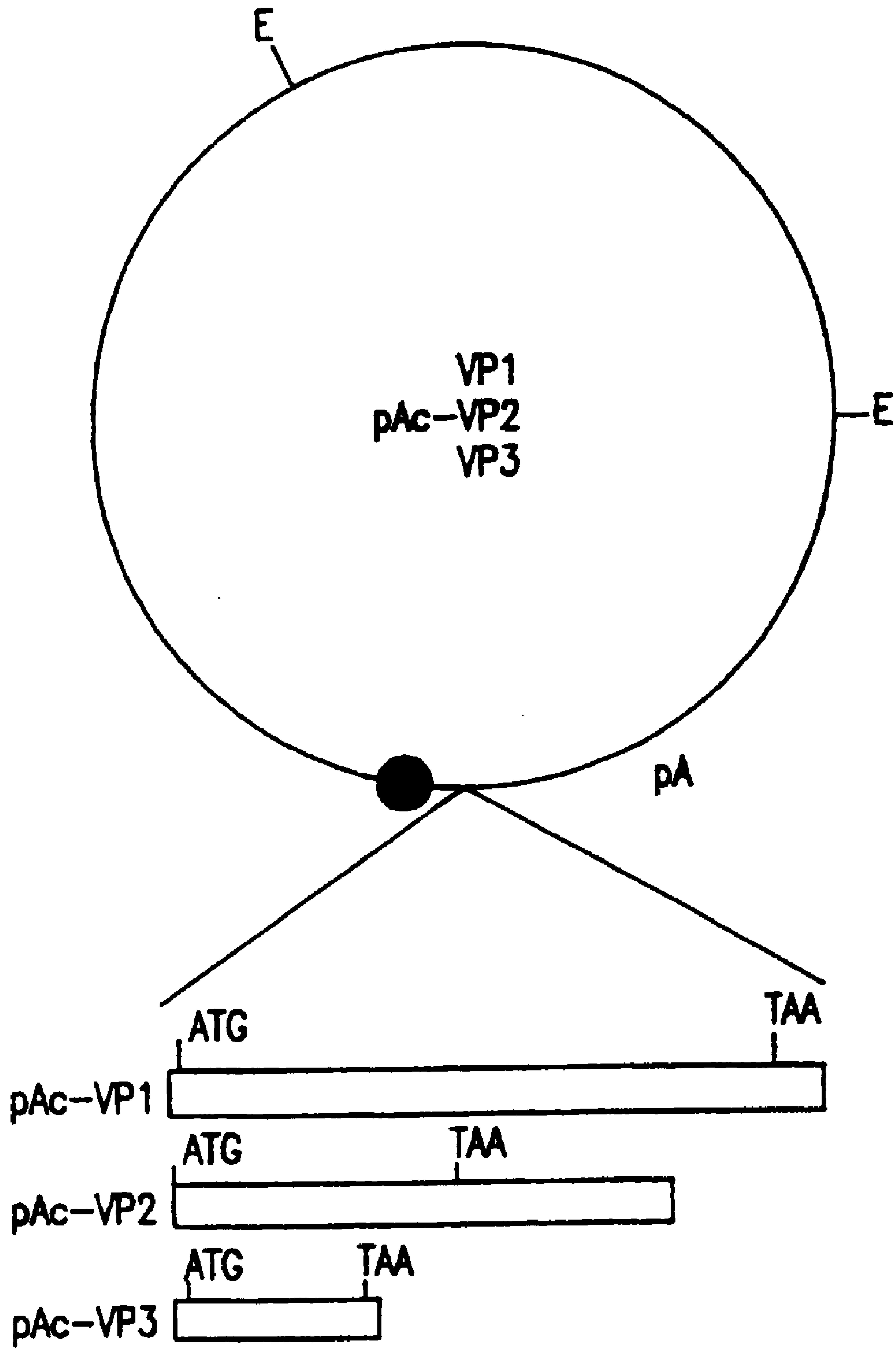
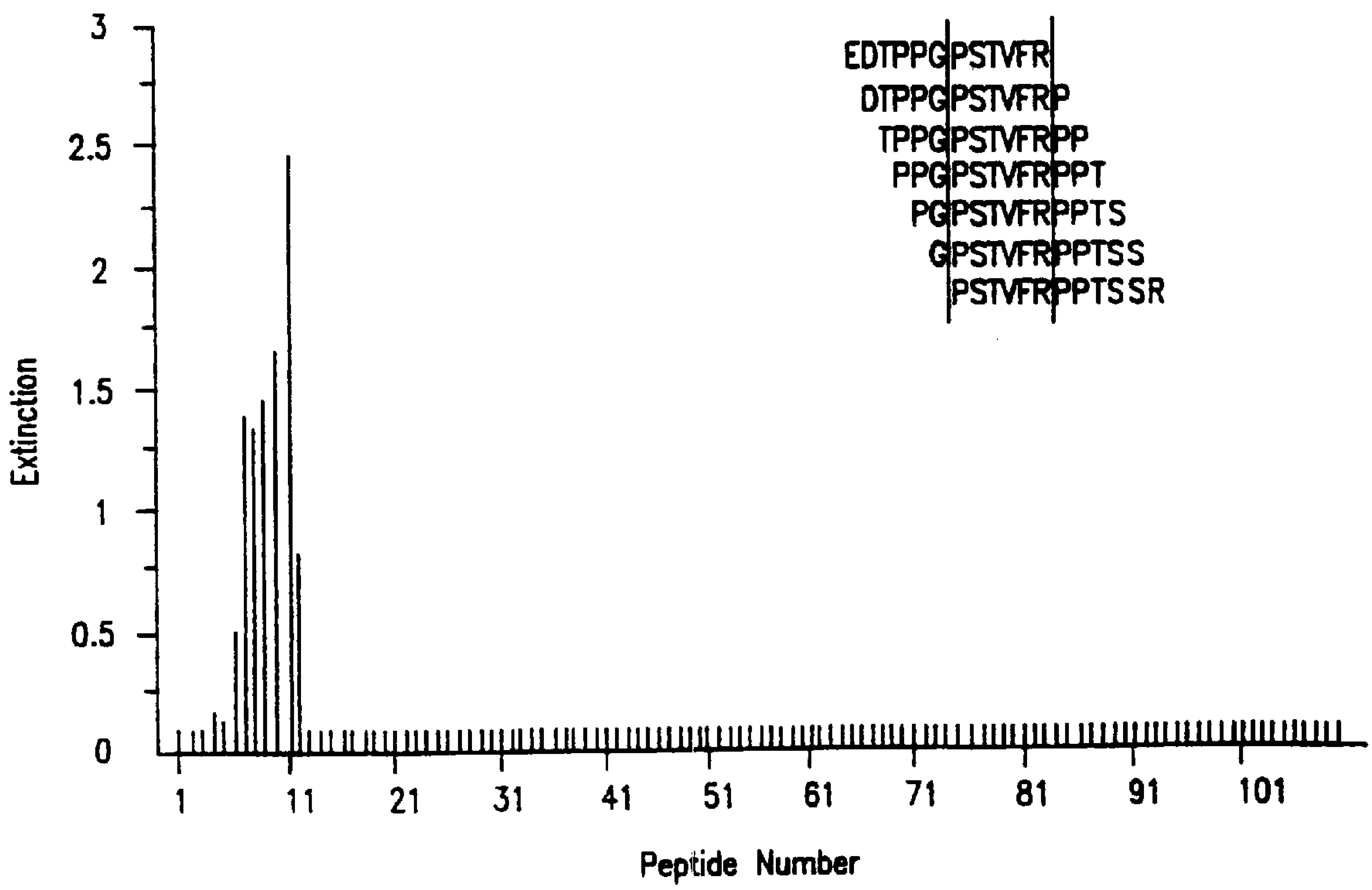
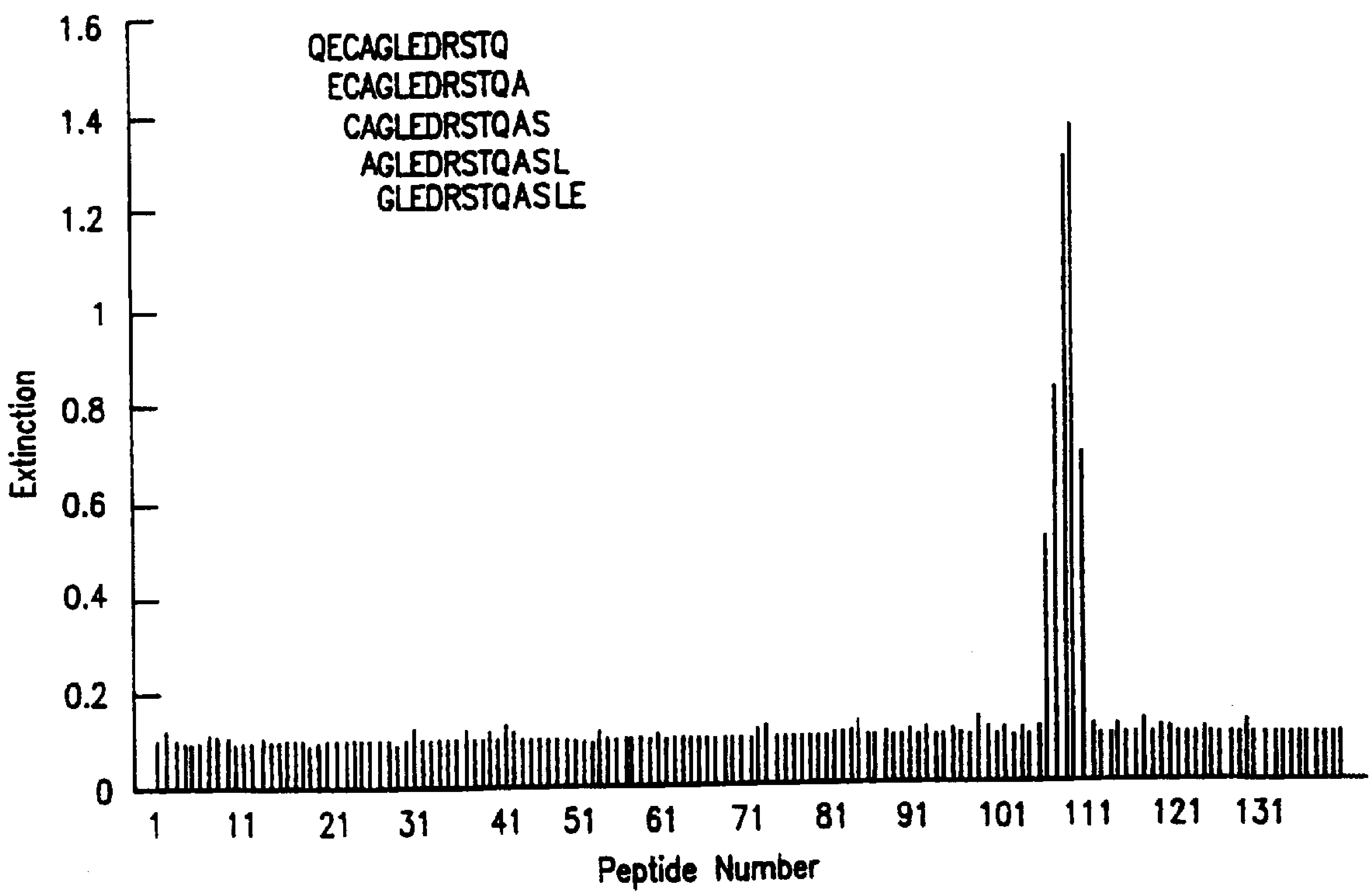

E
VP1
pAc-VP2
VP3
E
pA
ATG
TAA
pAc-VP1
ATG
TAA
pAc-VP2
ATG
TAA
pAc-VP3

FIG. 4



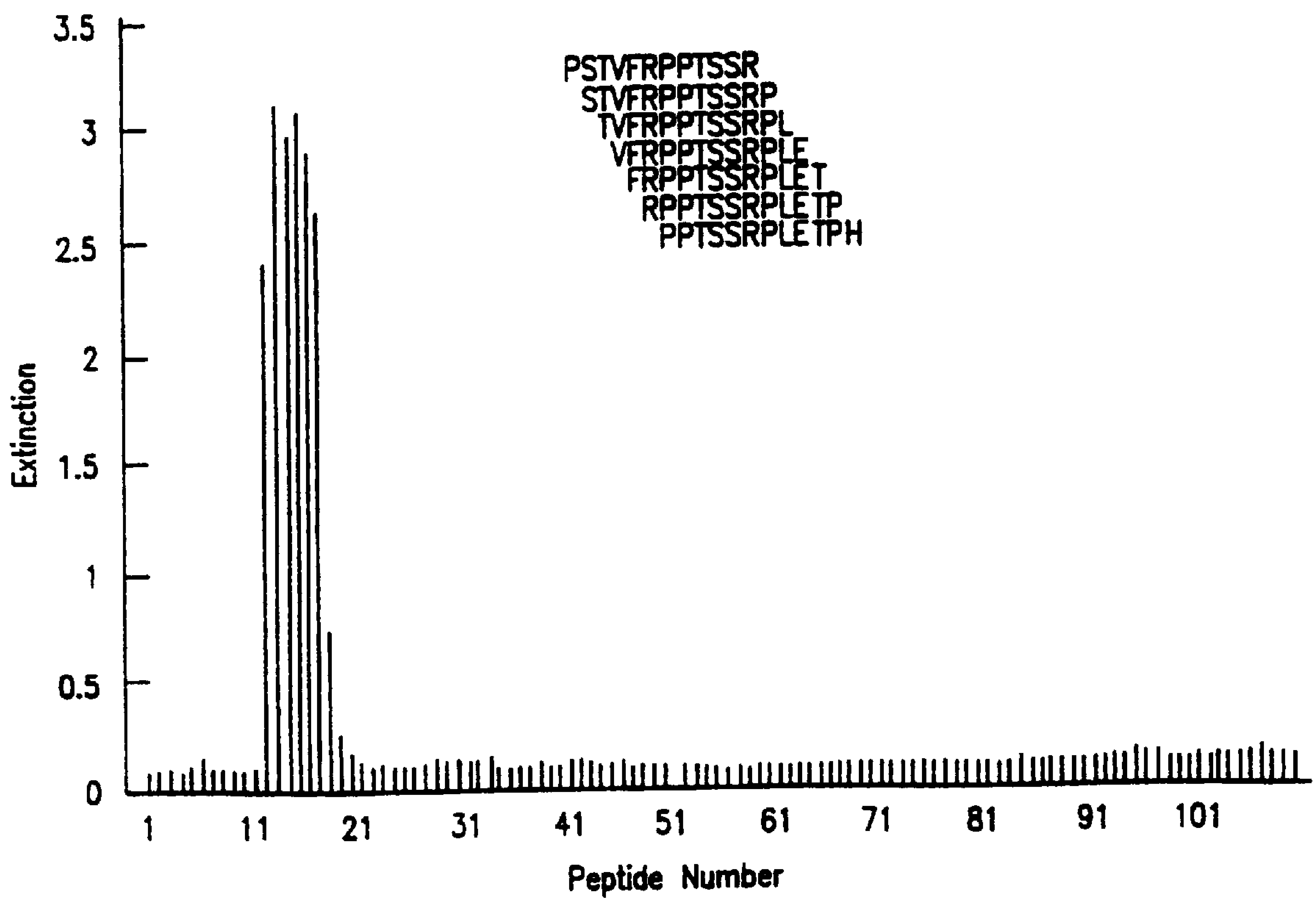
EDTPPGPSTVFR
DTPPGPSTVFRP
TPPGPSTVFRPP
PPGPSTVFRPPT
PGPSTVFRPPTS
GPSTVFRPPTSS
PSTVFRPPTSSR
Extinction
3
2.5
2
1.5
1
0.5
0
1
11
21
31
41
51
61
71
81
91
101
Peptide Number


FIG. 5



QECAGLEDRSTQ
ECAGLEDRSTQA
CAGLEDRSTQAS
AGLEDRSTQASL
GLEDRSTQASLE
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Extinction
1
11
21
31
41
51
61
71
81
91
101
111
121
131
Peptide Number


FIG. 6



3.5
3
2.5
2
1.5
1
0.5
0
Extinction
PSTVFRPPTSSR
STVFRPPTSSRP
TVFRPPTSSRPL
VFRPPTSSRPLE
FRPPTSSRPLET
RPPTSSRPLETP
PPTSSRPLETPH
1
11
21
31
41
51
61
71
81
91
101
Peptide Number


FIG. 7

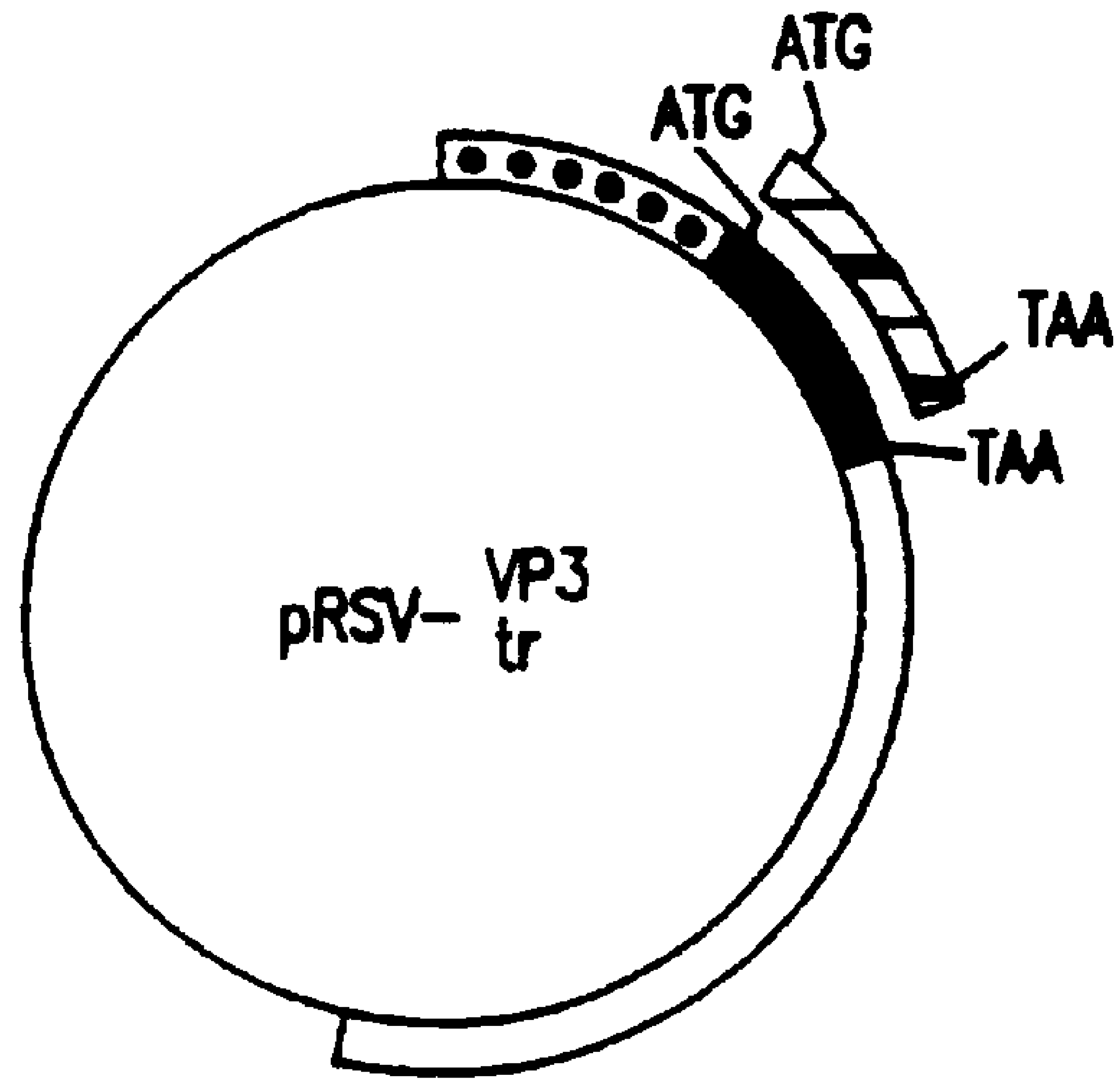



FIG. 8A

Amino-Acid Sequence of VP3.

1 -M N A L Q E D T P P G P S T V
F R P P T S S R P L E T P H C
R E I R I G I A G I T I T L S
L C G C A N A R A P T L R S A
T A D N S E S T G F K N V P D
L R T D Q P K P P S K K R S C
D P S E Y R V S E L K E S L I
T T T P S R P R T A K R R I R
L -121

FIG. 8B

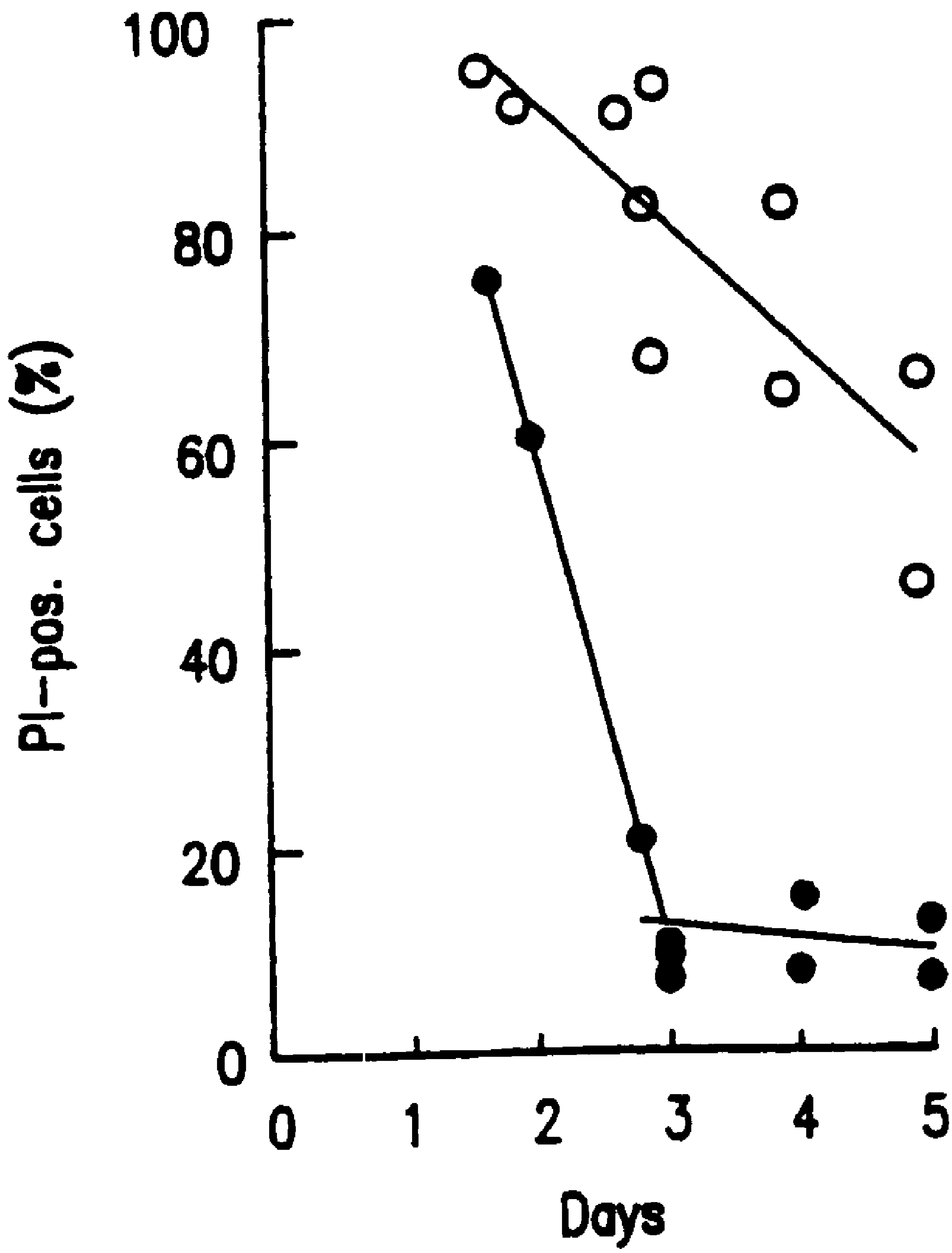



FIG. 9

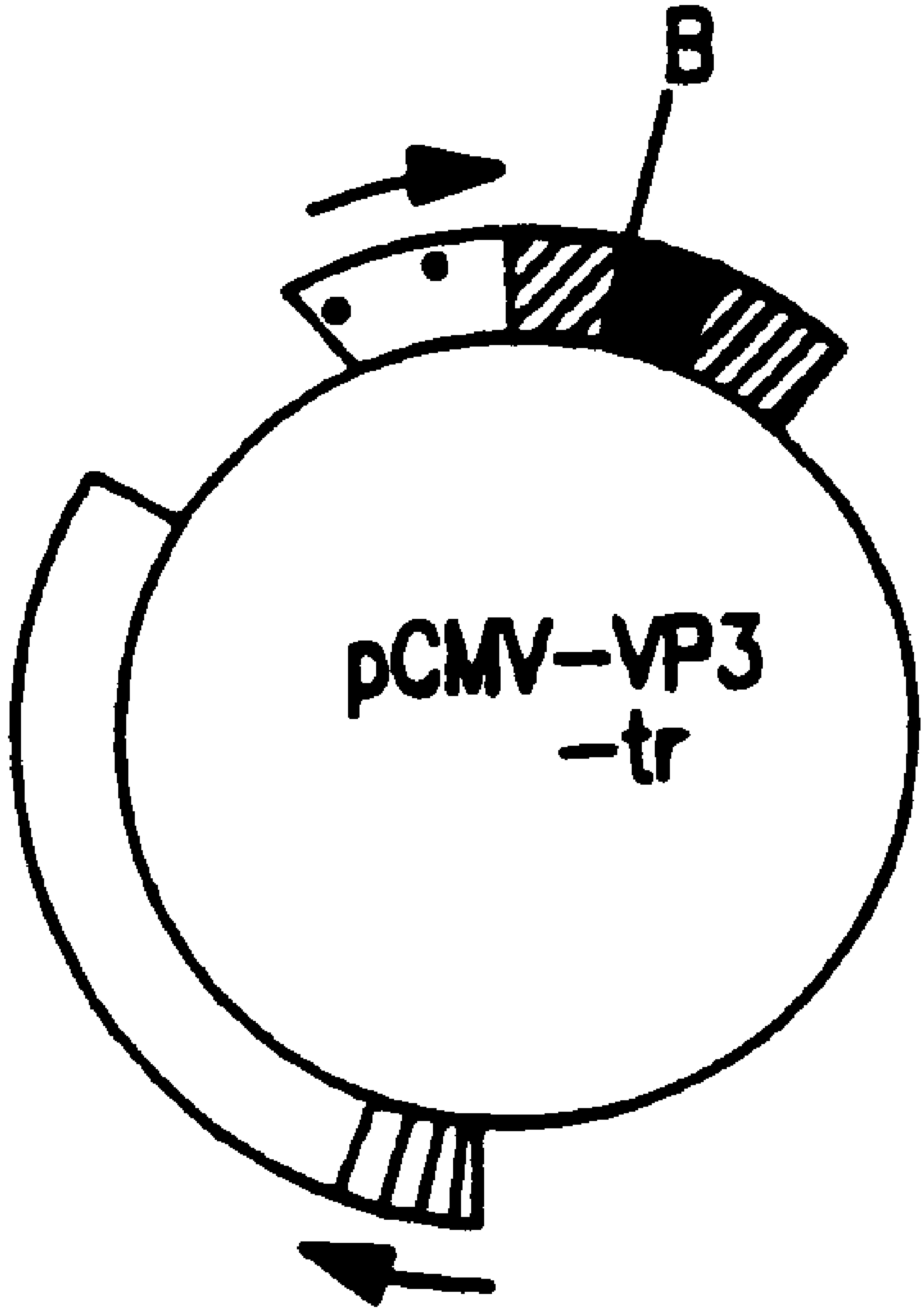



FIG. 10

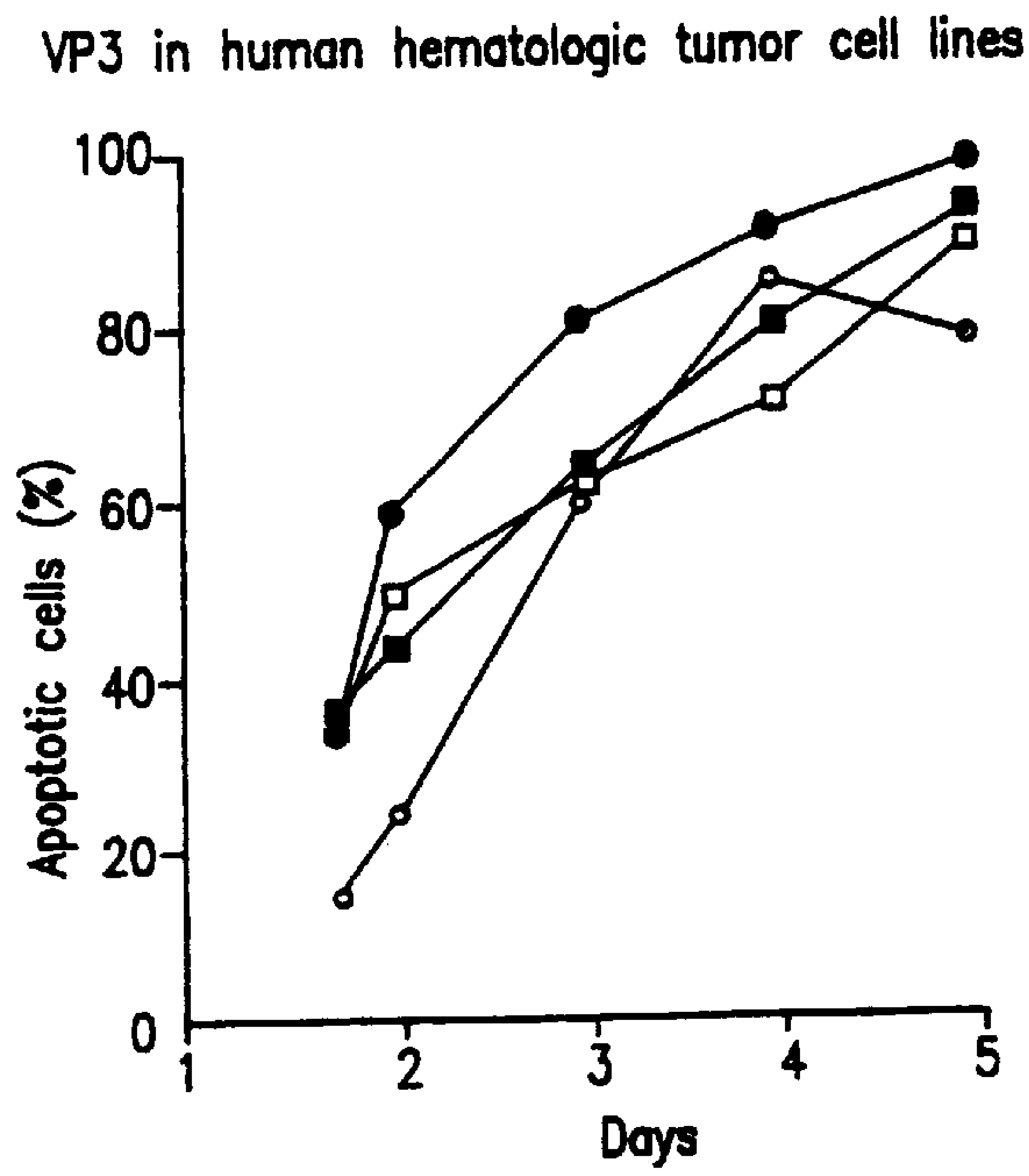



FIG. 11A

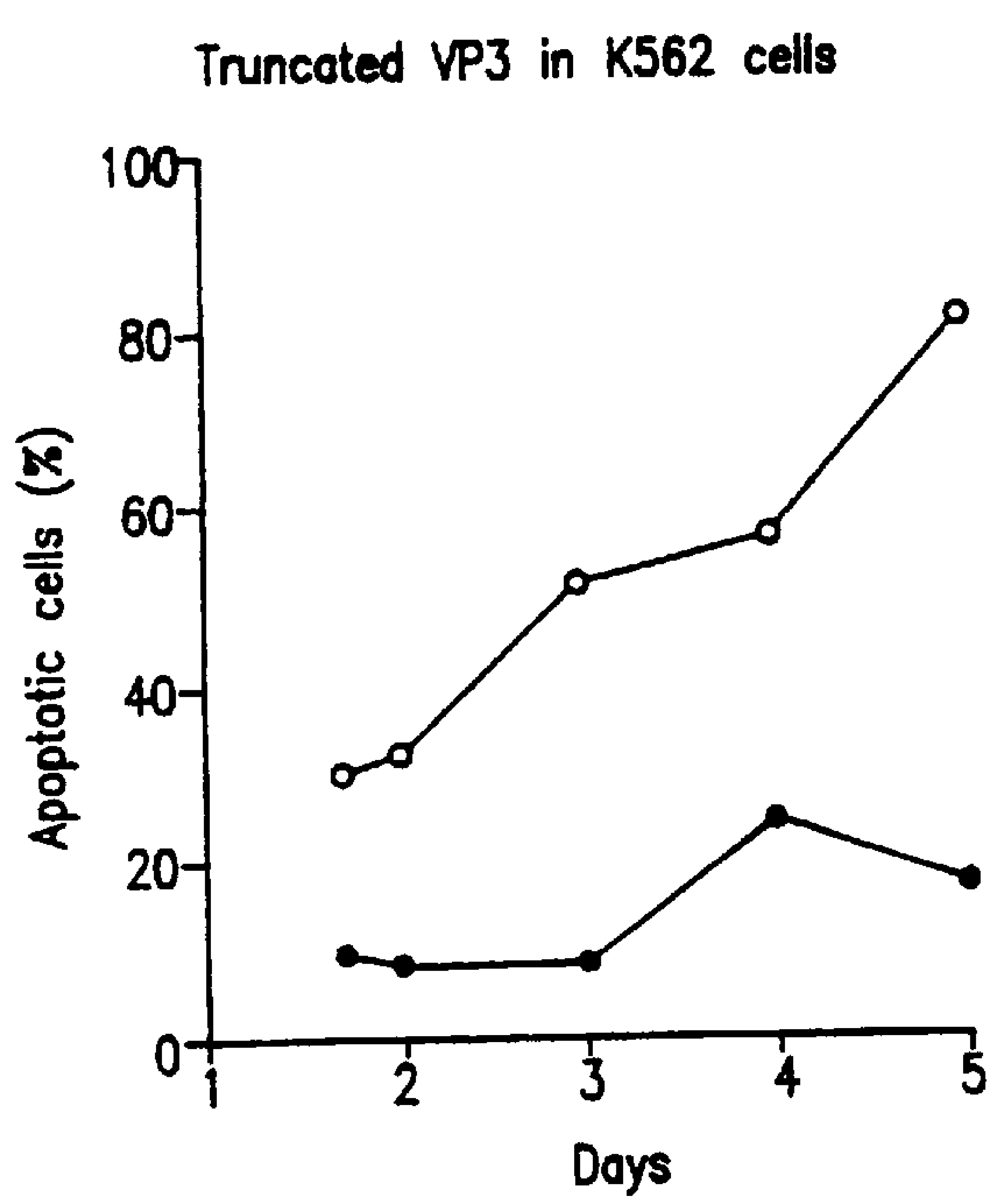



FIG. 11B

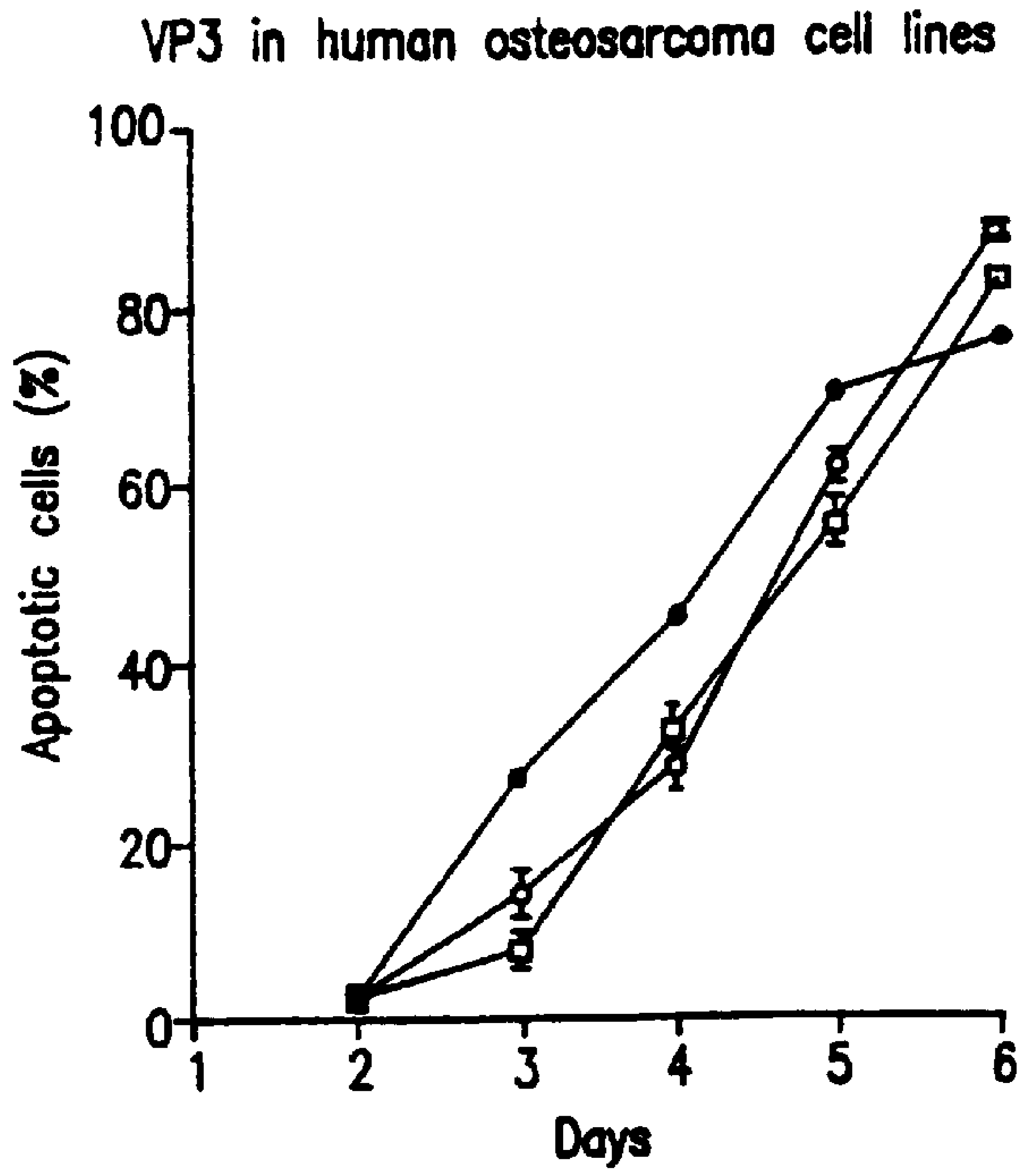



FIG. 12A

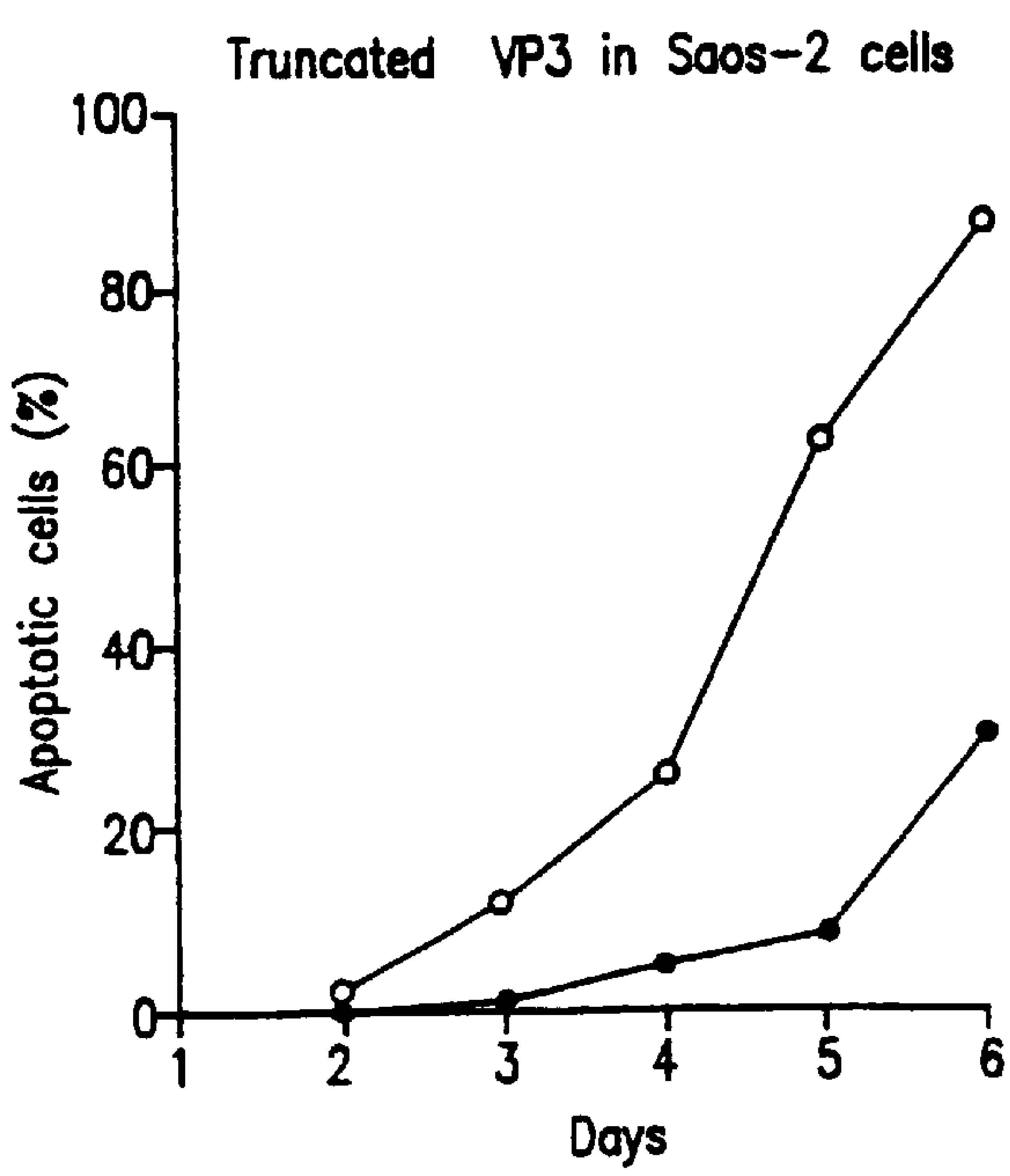



FIG. 12B

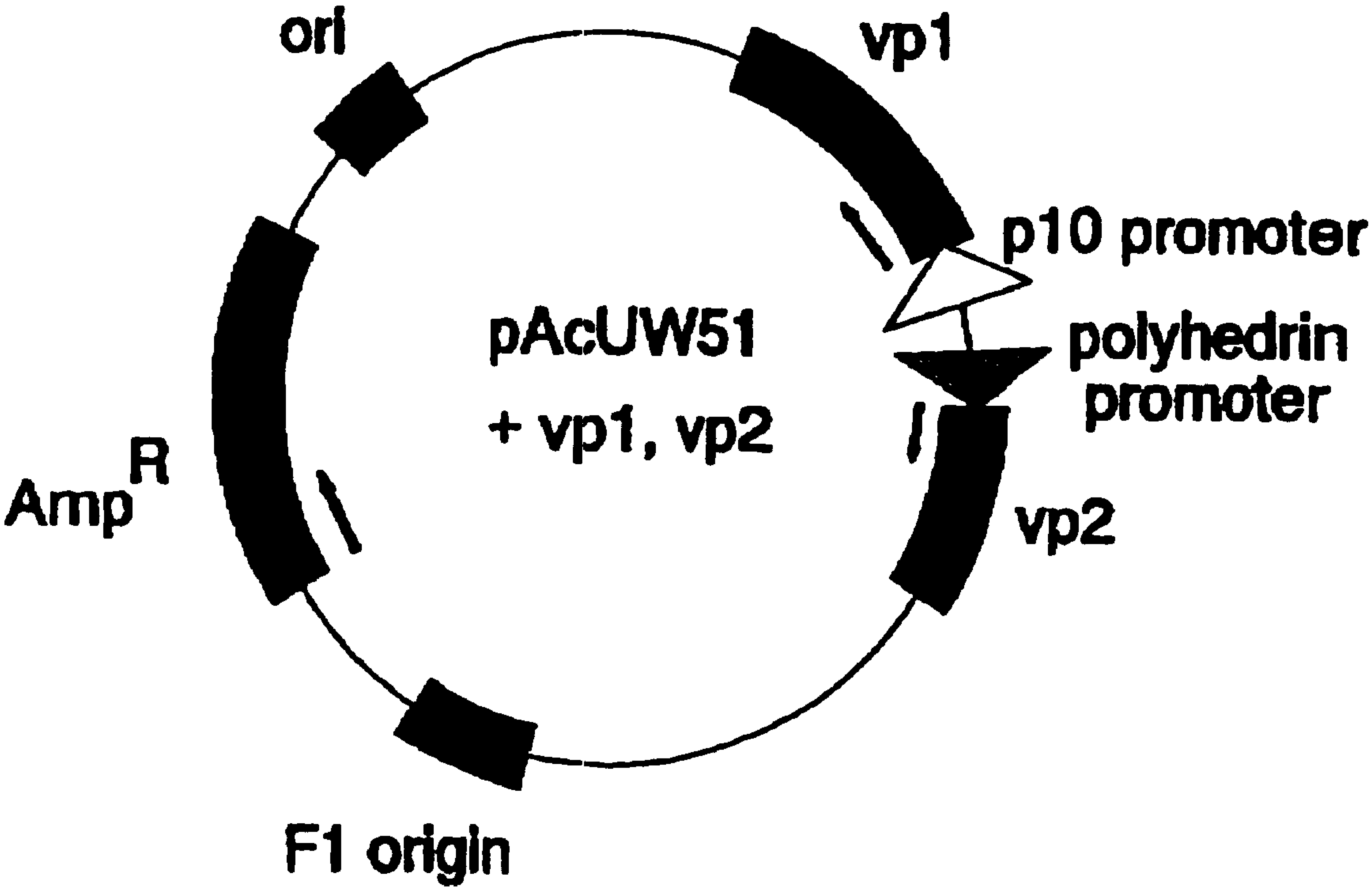
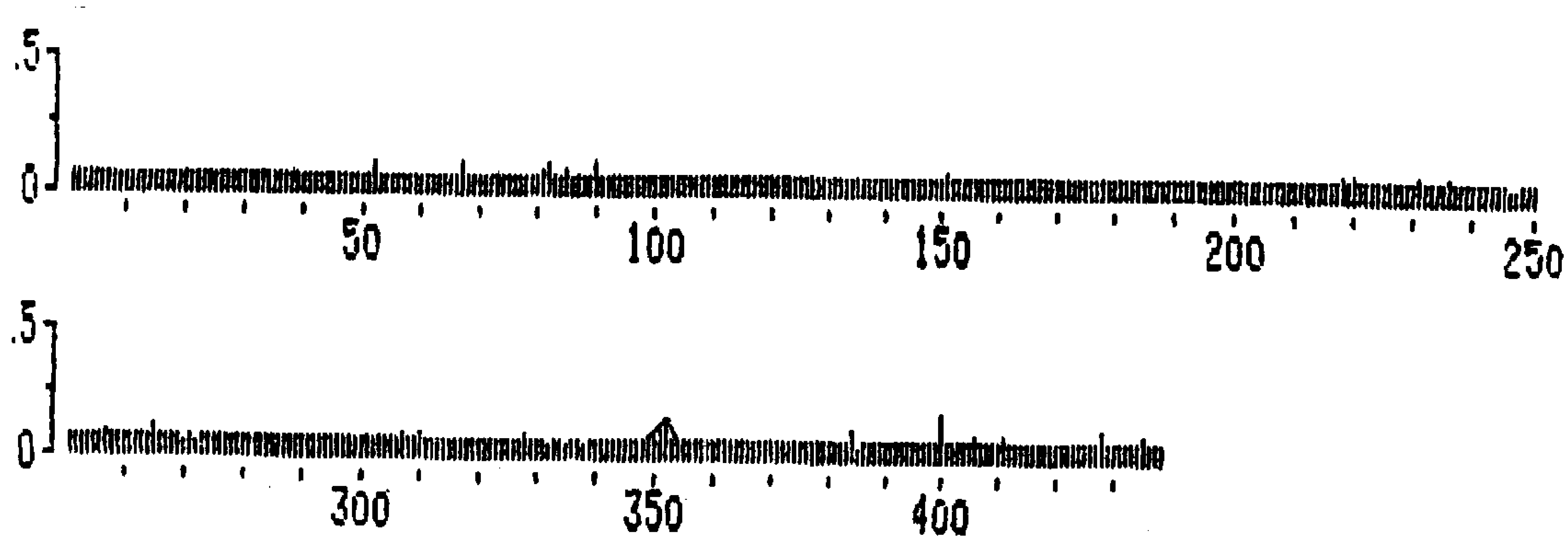

ori
vp1
p10 promoter
polyhedrin
promoter
pAcUW51
+ vp1, vp2
Amp$^R$
vp2
F1 origin

FIG. 13

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| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 88 | 86 | 83 | 83 | 89 | 86 | 85 | 85 | 105 | 81 | 88 | 91 | 83 | 81 | 91 |
| 83 | 92 | 86 | 83 | 86 | 121 | 86 | 86 | 135 | 83 | 86 | 92 | 86 | 80 | 81 |
| 86 | 88 | 83 | 86 | 97 | 88 | 86 | 86 | 83 | 86 | 92 | 93 | 86 | 83 | 86 |
| 92 | 85 | 86 | 86 | 93 | 85 | 86 | 86 | 86 | 85 | 88 | 81 | 85 | 81 | 83 |
| 88 | 88 | 89 | 83 | 83 | 83 | 88 | 88 | 101 | 86 | 95 | 83 | 86 | 81 | 83 |
| 93 | 92 | 83 | 88 | 85 | 93 | 96 | 88 | 81 | 88 | 93 | 81 | 85 | 81 | 81 |
| 93 | 92 | 85 | 86 | 98 | 83 | 138 | 88 | 83 | 89 | 92 | 83 | 83 | 86 | 83 |
| 93 | 83 | 86 | 85 | 86 | 83 | 85 | 83 | 86 | 85 | 93 | 83 | 81 | 83 | 83 |
| 91 | 88 | 89 | 86 | 86 | 83 | 86 | 93 | 86 | 86 | 93 | 80 | 81 | 83 | 86 |
| 88 | 83 | 86 | 86 | 86 | 86 | 83 | 81 | 122 | 88 | 88 | 83 | 83 | 93 | 86 |

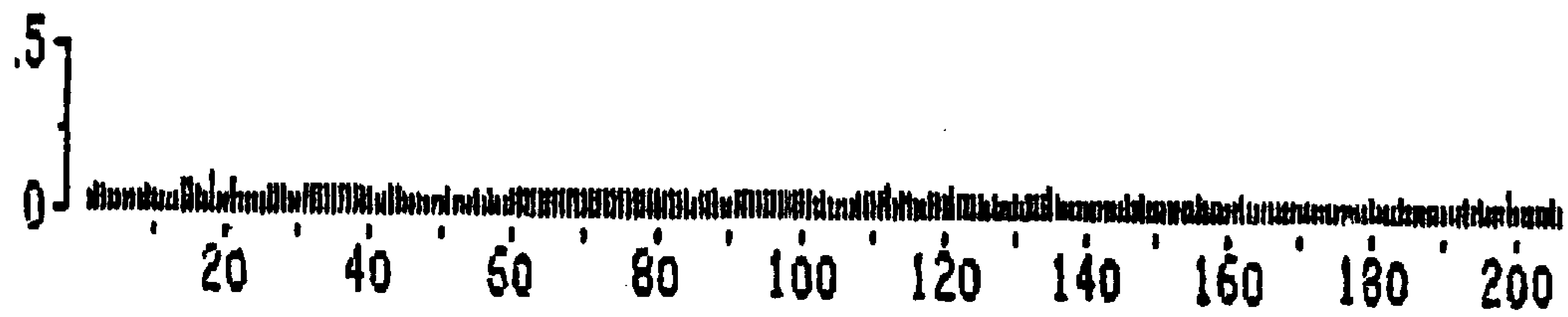
151 – 300

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 114 | 85 | 86 | 85 | 81 | 93 | 83 | 85 | 116 | 81 | 80 | 81 | 81 | 85 | 86 |
| 90 | 86 | 88 | 81 | 86 | 93 | 83 | 86 | 86 | 83 | 81 | 85 | 78 | 83 | 83 |
| 83 | 83 | 86 | 83 | 88 | 91 | 83 | 83 | 81 | 81 | 83 | 83 | 81 | 83 | 83 |
| 83 | 88 | 83 | 85 | 86 | 95 | 88 | 83 | 83 | 85 | 81 | 86 | 83 | 91 | 81 |
| 81 | 83 | 86 | 85 | 88 | 95 | 80 | 81 | 86 | 97 | 85 | 123 | 81 | 83 | 85 |
| 83 | 93 | 83 | 83 | 86 | 91 | 89 | 86 | 106 | 76 | 83 | 86 | 83 | 81 | 86 |
| 83 | 83 | 81 | 83 | 88 | 93 | 85 | 81 | 81 | 73 | 116 | 88 | 85 | 81 | 85 |
| 81 | 86 | 81 | 83 | 93 | 92 | 108 | 86 | 81 | 81 | 85 | 86 | 81 | 83 | 86 |
| 83 | 86 | 83 | 85 | 93 | 93 | 85 | 81 | 80 | 80 | 86 | 85 | 83 | 81 | 89 |
| 93 | 85 | 83 | 86 | 93 | 85 | 103 | 83 | 86 | 61 | 86 | 78 | 86 | 81 | 91 |

301 – 450

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 88 | 83 | 85 | 83 | 91 | 129 | 85 | 81 | 83 | 86 | 86 | 101 | 86 | 81 |
| 89 | 83 | 83 | 78 | 88 | 176 | 85 | 66 | 83 | 85 | 83 | 86 | 83 | 83 |
| 88 | 83 | 81 | 83 | 85 | 86 | 86 | 86 | 80 | 88 | 86 | 88 | 83 | 65 |
| 89 | 85 | 83 | 65 | 83 | 88 | 88 | 81 | 126 | 89 | 81 | 86 | 86 | 81 |
| 88 | 88 | 83 | 76 | 83 | 88 | 93 | 83 | 78 | 88 | 88 | 83 | 86 | 101 |
| 83 | 86 | 83 | 83 | 83 | 86 | 86 | 85 | 83 | 88 | 102 | 83 | 86 | 86 |
| 119 | 86 | 83 | 83 | 83 | 86 | 83 | 86 | 83 | 88 | 89 | 89 | 89 | 86 |
| 81 | 81 | 104 | 78 | 88 | 86 | 83 | 86 | 83 | 86 | 89 | 86 | 136 | 86 |
| 86 | 83 | 86 | 83 | 86 | 83 | 99 | 85 | 85 | 85 | 95 | 88 | 86 | |
| 119 | 81 | 83 | 85 | 104 | 86 | 83 | 83 | 85 | 192 | 86 | 65 | 88 | |

FIG. 14

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| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
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| 91 | 73 | 76 | 81 | 78 | 71 | 81 | 91 | 81 | 81 | 78 | 104 | 92 | 85 | 76 |
| 85 | 76 | 78 | 83 | 95 | 78 | 83 | 80 | 83 | 80 | 95 | 75 | 85 | 96 | 78 |
| 76 | 104 | 79 | 83 | 83 | 78 | 83 | 81 | 81 | 81 | 76 | 98 | 93 | 81 | 78 |
| 71 | 106 | 78 | 83 | 78 | 81 | 81 | 86 | 78 | 86 | 73 | 91 | 80 | 102 | 76 |
| 71 | 80 | 83 | 81 | 76 | 78 | 83 | 81 | 80 | 81 | 76 | 76 | 78 | 90 | 83 |
| 73 | 76 | 98 | 81 | 78 | 80 | 83 | 80 | 81 | 91 | 73 | 78 | 80 | 78 | 76 |
| 71 | 133 | 80 | 80 | 73 | 73 | 81 | 83 | 80 | 99 | 93 | 81 | 76 | 78 | 80 |
| 96 | 75 | 71 | 85 | 78 | 78 | 83 | 83 | 78 | 81 | 93 | 83 | 78 | 78 | 78 |
| 73 | 76 | 78 | 80 | 78 | 80 | 83 | 81 | 79 | 81 | 86 | 92 | 81 | 78 | 78 |

151 – 300

| | | | | | |
|---|---|---|---|---|---|
| 78 | 75 | 73 | 76 | 76 | 70 |
| 78 | 81 | 78 | 73 | 76 | 76 |
| 76 | 78 | 71 | 78 | 83 | 75 |
| 78 | 78 | 76 | 80 | 71 | 73 |
| 76 | 78 | 75 | 73 | 86 | 80 |
| 80 | 75 | 76 | 76 | 78 | 78 |
| 78 | 73 | 73 | 76 | 76 | |
| 78 | 73 | 76 | 76 | 78 | |
| 78 | 68 | 76 | 76 | 103 | |
| 76 | 73 | 81 | 76 | 71 | |

FIG. 15

# CHICKEN ANEMIA VIRUS MUTANTS AND VACCINES AND USES BASED ON THE VIRAL PROTEINS VP1, VP2 AND VP3 OR SEQUENCES OF THAT VIRUS CODING THEREFOR

## CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of U.S. application Ser. No. 08/454,121, filed Nov. 30, 1995, which was the National Stage of International Application No. PCT/NL94/00168, filed Jul. 19, 1994; which was a continuation-in-part of U.S. application Ser. No. 08/030,335, filed Mar. 8, 1993, U.S. Pat. No. 5,491,073, which was the National Stage of International Application No. PCT/NL91/00165, filed Sep. 11, 1991. This application is related to U.S. application Ser. No. 08/485,001, filed Jun. 7, 1995, U.S. Pat. No. 5,981,502 and U.S. application Ser. No. 08/489,666, filed Jun. 7, 1995 U.S. Pat. No. 5,922,600.

## INTRODUCTION

1. Technical Field

The present invention relates to novel proteins and/or polypeptides of the Chicken Anemia Virus (CAV) together with vaccines and compositions for preventing or treating virus infections in poultry, in particular infections with CAV.

2. Background

Day-old chicks are most susceptible to CAV infections. In these animals lethargy, anorexia and anemia are observed from 10 days after inoculation with CAV. After infection mortality may increase to a maximum of 50%. With increasing age the resistance also increases. Jeurissen et al. (1992) supra have reported that only the hematocrit values of chicks that had been infected with CAV at an age of 1–3 days are decreased. CAV infections of 1–21 days old chicks result in a depletion of in particular the thymus cortex. However, in older chickens CAV can subclinically multiply. CAV infection in older chickens can be determined by the occurrence of serum conversion (McIlroy et al., (1992) Avian Diseases 36:566–574).

The spread of CAV within a flock of chickens substantially occurs via contact infection. Most probable is ingestion of feces or other material contaminated with feces from CAV infected animals. Infection via the air, however, cannot be ruled out. Transmission of viruses to offspring via the egg is suggested by Yuasa et al., (1979) Avian Diseases 23:366–385 but this way of experimental vertical transmission of CAV from mother animals to chicks could not be demonstrated by us.

Immune deficiency resulting from the CAV induced deletion of the thymus cortex is considered to be the cause of disease symptoms occurring after secondary infections of normally non-pathogenic agents (De Boer et al., (1992) In: Proceedings World's Poultry Congress Symposium, Amsterdam, The Netherlands, 1:262–271); Avian Diseases 33:707–713; Engström, (1988) Avian Pathology 17:23–32; Rosenberger and Cloud, (1989); Von Bülow et al., (1986) J. Vet. Med. B 33:717–726; Yuasa et al., (1980) Avian Diseases 24:202–209). Thus CAV is isolated in animals with Newcastle disease, Marek's disease, infectious bursitis (Gumboro) and in animals with 'blue wing disease' in association with retroviruses. CAV infections lead to increased inoculation reactions, e.g. against Newcastle disease virus.

Maternal antibodies have been found to give an important protection against CAV infection. A recent study under



laboratory conditions has shown that maternal immune day-old chicks develop no CAV infection. Day-old chicks can also be protected passively by intravenous injection of antibodies from egg yolks of immune mother animals.

CAV can be multiplied in tissue culture, however, in general the titers so obtained are low. At present MDCC-MSB1 cells (Yuasa, (1983) National Institute of Animal Health Quarterly 23:13–20; Yuasa et al., (1983) ibid, 78–81) are used therefor, in which CAV induces a cytopathogenic effect 48–72 hours after infection. MDCC-MSB1 cells are also used to determine neutralizing antibodies and antibodies directed against CAV by means of immunofluorescence (Von Bülow et al., (1985) J. Vet. Medicine B 32:679–693; Chettle et al., (1991) The Veterinary Record 128:304–306). It has not been found possible so far to attenuate the virulence of CAV by serial passage in MDCC-MSB1 cells.

Older animals do not develop disease symptoms after CAV infection and chicks with maternal antibodies are protected. These data were used in Germany in a vaccination program based on controlled exposure to CAV of 14–16 weeks old mother animals. In the Netherlands this vaccination method is not allowed except at an experimental level because of the attendant risks. As mentioned above, it is quite possible that CAV can be transmitted to offspring via the fertilized egg. McNulty et al. (1991) Avian Diseases 35:263–268 have recently shown that flocks that are CAV seropositive have production numbers inferior to those of CAV seronegative flocks. Moreover, immune deficiency in chickens having a subclinical CAV infection has been shown. The possible vertical virus spread and the immune deficiency caused by CAV with (sub)clinical infections renders a control program based on an innocuous vaccine very desirable.

The Chicken Anemia Virus (CAV) is a recently characterized DNA virus (Noteborn and De Boer, (1990) Dutch Patent No. 9002008). It belongs to a new virus family. In young chickens CAV causes anemia by destruction of erythroblastoid precursor cells and immune deficiency by depletion of thymocytes. Lesions occur in the spleen and liver (Jeurissen et al., (1989) Thymus 14:115–123). A recent study has shown that the depletion of thymocytes is caused via apoptosis induced by CAV (Jeurissen et al., (1992) J. Virology 66:7383–7388).

Gelderblom et al. (1989) Archives of Virology 109:115–120 and Todd et al. (1990) J. Gen. Virology 71:819–823 have shown by means of electron microscopic studies that CAV particles have a T3 icosahedron symmetry and a diameter of 23–25 nm. The CAV particles concentrate after equilibrium sedimentation at a density of 1.33–1.34 g/ml in CsCl.

Todd et al., (1990) supra have shown that isolated virus particles contain only one protein having a molecular weight of 50 kDa. The single-stranded DNA in the CAV particles is in the form of a circular minus strand (Gelderblom et al., (1989, supra; Todd et al., (1990) supra; Noteborn et al., (1991) J. Virology 65:3131–3139). The replicative DNA intermediary was cloned and fully sequenced. The CAV genome is 2319 nucleotides long. On the basis of the genome structure and the DNA sequence the virus cannot be placed into one of the known virus families (Noteborn et al., (1991) supra; Todd et al., (1991) Archives Virology 71:819–823). The CAV genome contains three large, partially or completely overlapping reading frames coding for possible proteins having molecular weights of 51.6, 24.0 and 13.3 kDa. The CAV genome moreover contains one evident promoter/enhancer region and only one polyadenylation signal. Transcription of the replicative DNA intermediary produces a polyadenylated polycistronic RNA molecule of approximately 2100 nucleotides (Noteborn et al., (1992) supra).

## SUMMARY

Provided are methods and compositions derived from the Chicken Anemia Virus (CAV) for use in vaccines and other therapeutics, for example. The method of vaccinating host animals against CAV includes induction of neutralized antibodies by way of providing recombinantly produced VP1/VP2 compositions.

Besides, the invention relates to uses of the proteins of the CAV in the induction of apoptosis (programmed cell death). In particular, the proteins (polypeptides) can be used in the induction of apoptosis in tumor cells.

Besides, the proteins according to the invention can also be used in the elimination of other undesired cell populations, such as autoimmune reactive T cells in autoimmune disease, such as rheumatoid arthritis, lupus, etc.

The invention further provides for the induction of cell death by means of gene therapy. Processes for preparing these therapeutics and processes for treatment, therewith are also subjects of the invention.

The Chicken Anemia Virus (CAV) is a recently characterized DNA virus (Noteborn and De Boer, 1990). It belongs to a new virus family. In young chickens CAV causes anemia by destruction of erythroblastoid precursor cells and immune deficiency by depletion of thymocytes. Lesions occur in the spleen and liver (Jeurissen et al., 1989). A recent study has shown that the depletion of thymocytes is caused via apoptosis induced by CAV ((Jeurissen et al., 1992b).

## BRIEF DESCRIPTION OF THE DRAWINGS

Description of the Figures

FIG. **1** (SEQ ID NOS:3 and 4) gives the DNA sequence and the amino acid sequence of the VP1 protein of Chicken Anemia Virus. The numbering of the CAV DNA sequences is as given in Dutch patent no. 9002008.

FIG. **2** (SEQ ID NOS:5 and 6) gives the DNA sequence and the amino acid sequence of the VP2 protein of Chicken Anemia Virus. The numbering of the CAV DNA sequences is as given in Dutch patent no. 9002008.

FIG. **3** (SEQ ID NOS:7 and 8) gives the DNA sequence and the amino acid sequence of the VP3 protein of Chicken Anemia Virus. The numbering of the CAV DNA sequences is as given in Dutch patent no. 9002008.

FIG. **4** shows the diagrammatic representation of the 3 CAV recombinant transfer vectors pAc-VP1, pAc-VP2 and pAc-VP3. ●=polyhedron promoter, ATG=initiation codon, pA=polyadenylation signal, E=EcoRI.

FIG. **5** (SEQ ID NOS:9–15) shows the pepscan analysis of the monoclonal antibody CVI-CAV-85.1 with peptides (12-mers) derived from VP3. The core sequence PSTVFR (SEQ ID NO:28) against which the monoclonal CVI-CAV-85.1 is directed, is at positions 12 to 17 of the VP3 amino acid sequence (Noteborn et al., (1991).

FIG. **6** (SEQ ID NOS:16–20) shows the pepscan analysis of the monoclonal antibody 111.2 with peptides (12-mers) derived from VP2. Monoclonal 111.2 is directed against the epitope GLEDRSTQ (SEQ ID NO:29) which is at positions 109 to 116 of the VP2 amino acid sequence (Noteborn et al., (1991). Only the results obtained with peptides nos. 1 through 140 are shown (extinction of peptides nos. 141 through 206≦0.103).

FIG. **7** (SEQ ID NOS:21–27) shows the pepscan analysis of the monoclonal antibody 111.3 with peptides (12-mers) derived from VP3. Monoclonal 111.3 is directed against the epitope PTSSR (SEQ ID NO:30) which is at positions 19 to 23 of the VP3 amino acid sequence (Noteborn et al., (1991).

FIG. **8**, Panel A shows the diagrammatic representation of the 2 expression vectors pRSV-VP3 and pRSV-tr. ■=VP3, ▧=VP3tr, ▣=RSV LTR, □=SV40. Panel B (SEQ ID NO:7) shows the amino acid sequence of the CAV protein VP3. The proline residues are printed in italics and the basic amino acids in heavy type. The 11 C terminal amino acids, the codons of which are deleted in the expression vector, are underlined.

FIG. **9** shows the kinetics of the apoptotic effect of VP3 or truncated VP3. MDCC-MSB1 cells were transfected with plasmid pRSV-VP3 (●) or pRSV-tr (○), fixed and stained with the monoclonal antibody CVI-CAV-85.1 at different times after transfection. The percentages of the immunofluorescent cells with nuclei which normally stain with propidium iodide are given. Per experiment at least 100 cells were counted which had expressed VP3 or truncated VP3.

FIG. **10** shows the diagrammatic representation of the expression vectors pCMV-VP3 and pCMV-tr ▣=CMV promoter, ▨=rabbit B-globin, □=neomycin resistance, ■=VP3 or truncated VP3, ▥=RSV promoter, _=pBR322 sequences, B=BamHI cloning site.

FIGS. **11**A and **11**B show the kinetics of the apoptotic effect of VP3 on human hematopoietic (tumor) cells. The cell line KG1 was transfected with plasmid pRSV-VP3, and the cell lines DOHH-2, K562 and Jobo-0 were transfected with plasmid pCMV-VP3. The percentages of the VP3-positive cells with nuclei that weakly stain with propidium iodide, apoptotic cells, are given. Per experiment at least 200 cells were counted. For FIG. **11***a*: -○-=KG1, -●-=DOHH-2,-□-=K562, -■-=Jobo-O. For FIG. **11***b*: -○-=K562*pCMV-VP3, -●-=K562*pCMV-trVP3.

FIGS. **12**A and **12**B show the kinetics of the apoptotic effect of VP3 on human osteosarcoma cell lines. Cells of the cell lines Saos-2, Saos-2/Ala143 and U2-OS were transfected with plasmid pCMV-VP3. The percentages of the VP3-positive cells with nuclei that weakly stain with propidium iodide, apoptotic cells, are given. Per experiment at least 500 cells were counted. For FIG. **12***a*: -□-=Sa05-2/Algl43, mutant p53, -○-=Sa05-2, p53-, -●-=U2-05, p53t. For FIG. **12***b*: -○-=Sa05-2*pCMV-VP3, -●-=Sa05-2*pCMV-trVP3.

FIG. **13** shows the diagrammatic representation of the recombinant transfer vector pUW-VP1/VP2.

FIG. **14** shows the pepscan analysis of the neutralizing monoclonal antibodies of type 132.1 with peptides (12-mers) derived from VP1.

FIG. **15** shows the pepscan analysis of the neutralizing monoclonal antibodies of type 132.1 with peptides (12-mers) derived from VP2.

## DESCRIPTION OF SPECIFIC EMBODIMENTS

In particular, the invention related to vaccines that are less pathogenic than the CAV itself but yet lead to the generation of neutralizing antibodies in the immunized animal. Besides, the invention relates to compositions containing antibodies against parts of the CAV for controlling infections with CAV. Anti-idiotype antibodies which possess an immunogenicity corresponding with the antigen also are a subject of the invention. The invention also relates to antibodies for the detection or control of CAV infections. Diagnostic test kits for the detection of CAV also will be described. The invention further relates to recombinant DNA molecules derived from CAV, which code for at least an immunogenic part of a CAV protein and host cells transfected with such recombinant DNA molecules. Vaccines based on these host cells are made possible by this invention. So-called living virus vaccines, in which a piece of DNA coding for at least an immunogenic part of a CAV protein is brought into a virus that is infectious to the desired host also are a subject of the invention. Processes for the prophylaxis or control of CAV infections, in particular in chickens, and processes for the preparation of recombinant parts of CAV comprising sequences, and processes for the preparation of vaccines are also subjects of the invention. Besides, the invention relates to uses of the proteins of the CAV in the induction of apoptosis (programmed cell death). In particular, the proteins (polypeptides) can be used in the induction of apoptosis in tumor cells. Besides, the proteins according to the invention can also be used in the elimination of other undesired cell populations, such as autoimmune reactive T cells in autoimmune diseases, such a rheumatoid arthritis, lupus, etc. The invention further provides for the induction of cell death by means of gene therapy. Processes for preparing these therapeutics and processes for treatment therewith are also subjects of the invention.

In general, inactivated vaccines and subunit vaccines are the safest vaccines. The fact that under tissue culture conditions CAV multiplies only to low titers renders the preparation of an inactivated vaccine relatively expensive and laborious. For the preparation of a subunit vaccine against CAV infections those CAV proteins are necessary which induce a protective immune response in vaccinated chickens. Thus far only one protein (called VP1) has been found in purified CAV particles.

Surprisingly, it has now been found that this protein alone is not capable of giving an immune response that protects against CAV infections. It has been found that in spite of the fact that VP1 seems to be the only protein present in the virus particle, the VP2 protein now expressed by us for the first time is essential for generating virus neutralizing antibodies. Therefore, it is possible only now to develop an effective vaccine on the basis of parts of the virus.

We have cloned the three open reading frames present on the CAV genome into baculovirus vectors. The three CAV proteins VP1, VP2 and VP3 were expressed into Sf9 cells alone, in combination with one of the other CAV proteins or all three simultaneously by means of (co)-infection with recombinant CAV baculoviruses. Mother animals were injected with crude cell lysates which contained one or more CAV proteins. Only after immunization of chickens with antigen preparations containing proportional amounts of all three CAV proteins or containing essentially VP1 and VP2 and also some VP3, did neutralizing antibodies developed. Eggs of such animals contained maternal antibodies against CAV. Infection tests with offspring of vaccinated mother animals showed that at least the CAV proteins VP1 and VP2 are necessary for the induction of a protective immune response. Offspring of mother animals injected with all three CAV proteins were even better protected against infections with CAV. Injection into chickens with all three CAV proteins that had each individually been produced in Sf9 cells, induced few neutralizing antibodies against CAV. This implies that for an optimum induction of neutralizing antibodies against CAV two or three CAV proteins must be synthesized together in a host cell.

It is possible that fragments of two or three CAV proteins are already sufficient to effect a protective immune response against CAV infections. The recombinant CAV products, VP1+VP2 or VP1+VP2+VP3, which will be used for vaccination of laying-hens, can be synthesized by means of the baculovirus system. The CAV proteins can also be synthesized by means of other systems, such as bacterial or yeast cells, via retro (viral) infection or gene amplification (CHO-dhfr system). The fact that 2 or 3 proteins encoded by the open reading frames of the CAV genome can induce a protective immune response in chickens is also applicable to the development of living virus vectors. The coding sequences for VP1+VP2 or VP1+VP2+VP3 are then cloned into living virus vectors. It is also possible that one of the CAV proteins VP1, VP2 or VP3, separately, but then within the context of a living virus vector, is also suitable for the induction of a protective immune response against CAV infections. The expression of fragments of one or more above-mentioned CAV proteins by living virus vectors may be sufficient for the induction of a protective immune response.

In poultry, only living virus vectors which themselves show a good replication in the avian system can be used. Eligible for the use of viral vectors in chickens are, among other things: fowl pox virus, retroviral vectors, herpes virus vectors (Marek's virus and turkey herpes virus), adenoviruses and laryngotracheitis virus. It has been found that the induction of cell death as induced by CAV can essentially be attributed to VP3 and partly to VP2.

CAV induces apoptosis in infected thmocytes. It is possible that a CAV infection of (human) tumors also results in the cell death of the tumor cells.

In vitro the CAV protein VP3 is in itself capable of inducing apoptosis in chicken mononuclear tumor cells and in diverse human tumor cells.

Expression of the CAV protein can therefore also be used for the induction of cell death in (human) tumors by means of DNA transfection. Expression of VP3 in (tumor) cells may also take place by infecting the cells with (retro)viral vectors that contain a coding sequence for VP3. Aminisitration to cells of non-viral components (e.g. liposomes or transferring-derived vectors) containing VP3 proteins and/or coding sequences for VP3 is a further possibility for the expression/presence of VP3 in (tumor) cells.

The above-mentioned uses may also serve for the possible induction of cell death by expression in (tumor) cells of VP2 or VP2 and VP3.

The CAV proteins VP2 and/or VP3 can be used in treatments for reducing (human) tumor formation. This may take place, e.g., by injecting the proteins according to the invention directly into a solid tumor or couple the proteins to a ligand having affinity to a tumor associated antiligand. This coupling can be effected both chemically and (in case the ligand is also a protein) via making recombinant fusion protein.

The chemical coupling can be effected directly or via a spacer group. Optionally, an inert carrier molecule may be selected, such as an indifferent serum protein, to which both the ligand and the viral protein are attached, whether or not via a spacer group.

Examples of frequently proposed combinations of ligand-antiligand interactions are ligand-receptor pairs, such as EGF/receptor, IL-2/receptor, /T cell receptor, antibody/tumor-antigen, etc.

Preference is to be given to ligand-antiligand combinations that can be internalized by the cell. When a conjugate is selected, it can be advantageous to apply an intrinsic unstable group as a coupling between the viral protein and the ligand, so that the viral protein in the cell returns in native form. Not in all cases will it be necessary to select an internalizing combination. Tumor cells are metabolically active and will actively or passively take up substances, i.e. also the proteins according to the invention, via phagocytosis and/or pinocytosis.

It has meanwhile become sufficiently known that antibodies can be manipulated in such a manner that they generate no immune response but still recognize the desired antigen.

It will be briefly explained hereinafter how animal antibodies can be made suitable for human use (humanizing), but it may be clear that also adaptations of another type are possible.

In the first pace, it is possible to chemically remove the constant part from the antibody to be humanized, so as to prepare FAB, FAB'2 or still smaller fragments (Winter et al., 1990). In general, these fragments will at least be less immunogenic. Such fragments can also be prepared by means of recombinant DNA technology.

Besides, it is possible to replace the constant pails of animal antibodies by their human counterparts by means of recombinant DNA technology (Cabilly et al., 1984; Boss et al., 1984).

Besides, it is further possible to inoculate the antigen-binding domains of animal antibodies into antibodies of human origin (Winter et al., 1987).

Known tumor antigens against which antibodies have been generated are, e.g., CEA (carcinoembryonic antigen) and the like.

By deletion of the C terminal 11 amino acids of VP3 the induction of apoptosis by VP3 is strongly reduced. Consequently, the pathogenic activity of CAV can be drastically reduced by introduction of a stop codon into the C terminal region of VP3. The extra stop codon in the coding region for VP3 is introduced into the CAV clone pCAV/EcoRI (Noteborn and De Boer, Dutch Patent No. 9002008) which contains the complete CAV genome. The complete CAV mutant genome is cut from the vector and recycled. MDCC-MSB1 cells are transfected with the recycled CAV mutant DNA, and the virus offspring which are less pathogenic are harvested. Chickens are vaccinated with the attenuated CAV mutant viruses. Since the VP2 protein also has an effect on the induction of apoptosis, it is possible to also prepare attenuated CAV which contains a mutation in the coding region for VP2 or VP2 and VP3. The above-mentioned introduction of a stop codon into the coding region for VP2 and/or VP3 can also be used in the production of CAV recombinant living virus vectors.

Animals infected with CAV at an older age develop no clinical symptoms. Yet it seems that such infections may lead to great economic losses for the poultry industry. Immunization of animals with the above-described recombinant CAV products will lead to an active protection against the above-mentioned subclinical symptoms. The three CAV proteins which were expressed into the baculovirus system separately or in combination with one or two other CAV proteins can be used for tracing antibodies directed against CAV. Chickens infected or vaccinated with CAV can thus be traced. One or more CAV proteins can be used in immunoassays, such as enzyme-linked immunosorbent assay (ELISA), immunoperoxidase staining and immunofluorescence assay. For measuring neutralizing antibodies two or more CAV proteins are required.

Immunization of mice with the three CAV recombinant products synthesized in insect cells with CAV recombinant baculoviruses finally produced monoclonal antibodies specific for VP2 and VP3. These monoclonals reacted with specific structures in CAV infected calls and not with uninfected cells.

By means of the antibodies generated with recombinant CAV proteins, CAV proteins can be traced in organ preparations of CAV-infected chickens. On the basis of these data, reliable diagnostic tests can be developed. The monoclonal and polyclonal antibodies according to the invention also may be used in other diagnostic assays, such as ELISAs, RIAs, SPIAs, immunofluorescence assays and immunoperoxidase staining, optionally together with one or more CAV proteins or fragments thereof.

In principle, all known embodiments of immunological diagnostic tests are possible with all available labels, and depending on the test to be carried out and the conditions under which it must be carried out, a person of ordinary skill in the art will be able to select the most suitable embodiment. Besides, for the purpose of this invention antibodies and/or other proteins/polypeptides are also derivatives and/or fragments, as far as they possess the desired activity for use in an immunological diagnostic test. In the case of antibodies this means that they must at least be able to recognize the antigen.

The antibodies according to the invention also may be used for the passive immunization of poultry. Against the antibodies according to the invention antibodies can be generated which are a so-called "internal image" of the antigen and can thus be used as such again, in particular in passive immunizations and diagnostics.

The invention will be explained in more detail on the basis of the following experimental part. This is only for the purpose of illustration and should not be interpreted as a limitation of the scope of protection.

## EXAMPLES

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Materials and Methods

Chickens and Housing

Specific-pathogen-free (SPF) white leghorn strain A (WLA) chickens were obtained from the animal production facility of the DLO institute of Animal Science and Health, Lelystad, The Netherlands. The chickens were kept in conventional chicken houses and therefore vaccinated against Newcastle disease and infectious bronchitis at three weeks of age, for infectious bursal disease at four to five weeks of age, and revaccinated for bronchitis at 11 weeks of age and Newcastle disease at 13 weeks of age.

To obtain chicks with maternal antibodies directed against CAV, eggs of chickens immunized with recombinant CAV-proteins were collected and yolk extracts were tested for maternal antibodies in a CAV neutralization test. Shortly thereafter, fertilized eggs of animals that produced eggs with neutralizing antibodies were collected, incubated and transferred to modified Horsfall-Bauer isolators at hatch.

Baculovirus, insect cells and chicken T cells

The recombinant baculovirus pAcRP23-1acZ (Bishop, (1992) In: Baculovirus and recombinant protein production processes (Eds. Valk, et al. Editiones Roche, F. Hoffmann-La Roche Ltd., Basel, Switzerland) was obtained from Dr. R. Possee, NERC Institute of Virology, Oxford, England, and the genomic DNA was purified as described by the method of Summers and Smith (1987) Methods for Baculovirus Vectors and Insect Cell Culture Procedures. Texas Agricultural Experiment Station Bulletin No. 1555. *Spodoptera frugiperda* (Sf9) cells were obtained from the American Tissue Culture Collection (no. CRL 1711). Baculovirus stocks were grown in confluent monolayers and suspension cultures in TC-100 medium (Gibco/BRL) containing S-10% fetal calf serum as described by Summers and Smith (1987) supra.

The T cell line MDCC-MSB1 transformed with Marek's disease virus (Yuasa, (1983) National Institute of Animal Health Quarterly 23:13–20; Yuasa et al., (1983) National Institute of Animal Health Quarterly 23:78–81) was grown in RPMI-1680 medium (Gibco/BRL) containing 10% fetal calf serum; the cells were used for DNA transfection experiments.

Example 1

Recombinant Synthesis of CAV Protein

Cloning of CAV DNA: Construction of Recombinant CAV Transfer Vectors

All CAV DNA sequence are originally derived from the plasmid DNA pIc-20H/CAV-EcoRI (Noteborn and DeBoer, (1990), Dutch Patent No. 9002008). All cloning steps with plasmid DNA were in principle carried out according to the methods described by Maniatis et al. (1982) Molecular Cloning; A Laboratory Manual. New York: Cold Spring Harbor Laboratory.

The CAV genome contains three large open reading frames which partially or completely overlap each other. By using start codons in different reading frames the CAV genome codes for three unique proteins. The coding sequences for the CAV proteins were separately (VP1, FIG. **1**; VP2, FIG. **2**; and VP3, FIG. **3**) cloned into the baculovirus transfer vector pAcYM1. (Matsuura et al., (1987) J. General Virology 68:1233–1250), which was obtained from Dr. D. H. L. Bishop, NERC Institute of Virology, Oxford, England. Because the VP3 reading frame completely falls within the VP2 reading frame, VP3, in case of expression of VP2, is always synthesized too, though in a clearly lesser degree. The transfer vector pAcYM1 lacks the coding sequences for polyhedron, the polyhedron promoter inclusively contains the A-residue of the start codon for the polyhedron gene and the 3'-non-coding sequences including the polyadenylation signal. On both sides of the polyhedron sequences are flanking viral sequences. The transfer vector contains prokaryote sequences for multiplication in bacteria (Matsuura et al., (1987) supra).

The plasmid pEP-51.6 (Noteborn et al., (1992) *Gene*118:267–271) contains CAV DNA sequences of positions 791 to 2319. The CAV DNA insertion contains the complete coding region for the protein VP1 flanked by 62 bp 5'- an 117 bp 3'-non-coding DNA sequences. The plasmid pEP-51.6 was partially cut with HindIII, then completely cut with EcoRI, and the 'sticky ends' were filled by means of Klenow polymerase. A 1.53 kb CAV DNA fragment was isolated. The plasmid pAcYM1 was linearized with BamHI, the sticky ends filled by means of Klenow polymerase and finally treated with calf intestine alkaline phosphatase (CIP). The 1.53 kb CAV DNA fragment was ligated at the linearized pAcYM1 DNA. The orientation of VP1 in pAcYMI DNA was determined by restriction enzyme analysis, and the final construct pAcVP1 is shown in FIG. **4**.

To generate a recombinant transfer vector containing VP2-coating sequences, plasmid pEP-24.0, which contains the 1.15 kb BamHI DNA fragment with CAV DNA sequences of positions 354 to 1508 (Noteborn and De Boer, (1990) supra) was used. This CAV DNA fragment contains the coding region for VP2 flanked by 26 bp 5'- and 484 bp 3'-non-coding DNA sequences. 106 bp downstream of the start codon for VP2 the start codon for VP3 is found in another reading frame, and the other coding sequence for VP3. The plasmid pEP-24.0 was treated with BamHI; the 1.15 kb DNA fragment was isolated and ligated into at the BamHI linearized and CIP treated 9.3 kb pAcYM1 plasmid. The final DNA construct pAcVP2 was characterized with restriction enzymes and is shown in FIG. **4**.

To construct a transfer vector with sequences coding VP3, plasmid pEP-13.3 was used which contains the 0.46 kb BamHI-EcoRI DNA fragment with CAV DNA sequences of positions 427 to 868 (Noteborn and De Boer, (1990)). The CAV DNA fragment contains the coding region for VP3, 58 bp 5'- and 25 bp 3'-non-coding DNA sequences. Plasmid pEP-13.3 was cut with the restriction enzymes BamHI and EcoRI, and a 0.46 kb BamHI-EcoRI fragment was isolated. Transfer vector pAcYM1 DNA was linearized with BamHI and treated with CIP, and a 9.3 kb fragment was isolated. The two synthetic DNA oligomers 5'-GATCCAACCCGGGTTFG-3'(SEQ ID NO:1) and 5'-AATTCAACCCGGGTTG-3'(SEQ ID NO:2) were hybridized to each other and together form a BamHI-EcoRI DNA linker. The DNA linker was ligated at the 0.46 BamHI-EcoRI, and the 9.3 kb BamHI DNA fragment. The final construct pAc-VP3 was analyzed by restriction enzyme digestions and is shown in FIG. **4**.

DNA transformations were carried out in the *E. coli* strain HB101. All plasmids were multiplied in large cultures under agitation, purified on CsCl gradients, and then by filtration over Sephacryl S-500 columns.

DNA Transfection: Construction of Recombinant CAV Baculovirus

DNA of the recombinant baculovirus AcRP23-lacZ was isolated from extracellular baculoviruses according to a method described by Summers and Smith (1987) supra. The lacZ gene contains a unique cutting site for the restriction enzyme Bsu361. The AcRP23-lacZ was linearized by digestion with Bsu361. Sf9 cells were transfected with calcium phosphate precipitates of linearized baculovirus AcRP23 -lacZ DNA and recombinant transfer vector DNA according to the method of Smith et al. (1983) Mol. Cell Biol. 3:2156–2165; this is an adaptation of the transfection protocol of Graham and Van der Eb (1973) Virology 52:456–467 for Sf9 cells. Each of the three recombinant CAV transfer vectors was transfected separately, together with the recombinant baculovirus AcRP23-lacZ DNA, in Sf9 cells. Transfection occurred with "naked" baculovirus DNA and transfer vector DNA.

For the transfection of the diverse human and chicken cell lines 10 micrograms of pRSV-VP3, pCMV-VP3 pRSV-tr or pRSV-tr DNA were resuspended in 25 microliters of Milli-Q water and mixed with 260 microliters of TBS buffer. 15 microliters of 10 mg/ml DEAE dextran were added to the DNA mixture which was incubated for 30 minutes at room temperature. The cells were centrifuged at 1500 rpm in a table centrifuge. The medium was replaced by 5 ml TBS buffer, and the cells were carefully resuspended. The cells were pelleted, and the TBS buffer was removed. The cell pellet was carefully resuspended in 300 microliters of DEAE dextran/DNA mix and incubated for 30 minutes at room temperature. 0.5 ml 25% DMSO/TBS were added, and the suspension was incubated for 3 minutes at room temperature. 5 ml TBS were added, and the cells were centrifuged at 1500 rpm in a table centrifuge. The supernatant was removed, and 5 ml tissue medium were added. The cells were resuspended, centrifuged, taken up in 5 ml tissue culture medium and incubated at 37° C.-5% $CO_2$.

Selection of Recombinant CAV Baculovirus

The AcRP23-lacZ baculovirus genome contains, instead of the polyhedron gene, the lacZ gene, under the regulation of the polyhedron promoter. After homologous recombination baculoviruses were obtained which had always incorporated one of the three CAV genes instead of the lacZ gene and thus under regulation of the promoter of the polyhedron gene. The baculoviruses which have correctly incorporated the CAV gene no longer contain the lacZ gene. In the first instance, the recombinant CAV viruses were characterized for the absence of β-galactosidase activity in plaques of baculovirus infected insect cells. The supernatants containing extracellular baculoviruses were analyzed in a plaque essay with neutral red (Brown and Faulkner, (1977) J. Gen. Virol. 36:361–364) and X-gal (Brown et al., (1991) J. Virol. 65:2702–2706). The lacZ-negative plaques were inoculated on a monolayer of Sf9 cells in microtiter dishes. Five days after infection the supernatants were harvested and stored at 4° C.

The integration of CAV DNA sequences in the baculovirus genome was determined by means of a CAV-specific DNA probe in a hybridization experiment. The cell lysates were analyzed in a dot slot hybridization assay with 32p labeled pIc-20H/CAV-EcoRI DNA as a probe.

Expression of the CAV Proteins in Sf9 Cells

The expression of the specific CAV proteins in Sf9 cells infected with recombinant CAV was analyzed by protein labeling with $^{3}$H leucine and PAA-SDS gel electrophoresis. Monolayers of Sf9 cells were inoculated with supernatants of cell lysates which strongly hybridized with the labeled CAV DNA probe. Two days after infection the cells were labeled with $^{3}$H leucine. The proteins were separated on 14% polyacrylamide (PAA) SDS gels (Laemmli, (1970) Nature 227:680–685, made visible by means of a fluorography method and tested for the presence of specific recombinant CAV protein and the absence of the β-galactosidase protein.

Synthesis of Crude CAV Protein Preparations

Recombinant CAV baculoviruses which expressed the expected CAV protein in infected Sf9 cells, were prepared up according to the method described by Summers and Smith (1983) supra. Monolayers of Sf9 cells were infected with one type of recombinant CAV baculovirus having a multiplicity of infection (moi) of approximately 5 plaque-forming units (pfu) per cell. Co-infections of two or three different CAV recombinant baculoviruses were carried out on Sf9 cell monolayers having a moi of 10 pfu of each recombinant CAV baculovirus per cell. Three days after infection the infected Sf9 cells were harvested. The crude cell lysates were suspended in PBS buffer.

The CAV protein VP1 has a calculated molecular weight of 51.6 kDa (Noteborn and De Boer, (1990) supra). Lysates of insect cells infected with recombinant VP1 baculovirus contain a protein of 52 kDa in addition to baculoviral and cellular products. The 52 kDa protein was absent in lysates of insect cells infected with the baculovirus AcRP23-lacZ and in non-infected cells. In vitro expression of the coding sequence of VP1 resulted in a protein of 52 kDa (Noteborn and De Boer, (1992) supra). Most probably, VP1 is not glycosylated because VP1 which is synthesized in a rabbit reticulocyte lysate and VP1 synthesized in insect cells have the same molecular weight.

Translation of the gene coding for VP2, but also containing all coding sequences for VP3, produced in an in vitro system specific CAV proteins of 30 and 28 kDa and a minor amount of a 16 kDa protein product. Translation of only the open reading frame coding for VP3 in an in vitro system, however, produced only a protein of 16 kDa. Expression of VP2 by recombinant VP2 baculovirus in infected insect cells produced specific products of approximately 28 kDa and 30 kDa. Sf9 cells infected with a recombinant-lacZ baculovirus do not contain these CAV-specific proteins. The CAV-specific product of 16 kDa could mostly be demonstrated in very small amounts only. These data show that the recombinant VP2 baculovirus strongly expresses the protein VP2 and expresses VP3 in but a minor degree. A possible explanation thereof is that an internal start codon in a gene lying on the baculovirus genome is used very inefficiently.

Recombinant VP3 baculovirus synthesized in infected insect cells a main product of 16 kDa and small amounts of some proteins having molecular weights of approximately 21,000 and 12,000–14,000. In an immunofluorescence assay the CAV-specific monoclonal antibody CVI-CAV-85.1 reacted specifically with Sf9 cells expressing VP3. This monoclonal antibody precipitated specifically only a protein having a molecular weight of 16,000 from lysates of radioactively labeled Sf9 cells infected with VP3 recombinant baculovirus. In a pepscan analysis (Geysen et al., 1984) the epitope of the monoclonal antibody CVI-CAV-85.1 was localized on the N-terminus of VP3. The pepscan analysis is shown in FIG. **5**.

## Example 2

## Immunization of Chickens with CAV-Specific Proteins

Induction of Neutralizing Antibodies in Chickens Immunized with Recombinant CAV Proteins

In case of chicken anemia it has been determined that neutralizing antibodies properly correlate with protection. The CAV protein or several CAV proteins inducing neutralizing antibodies in chickens thus form the basis of a subunit vaccine.

In the first instance we have examined which CAV protein is capable of inducing neutralizing antibodies against CAV in chickens. Groups of 8 chickens at an age of approximately 6 weeks were injected with lysates of $10^{6}$ or $10^{8}$ recombinant CAV-infected Sf9 cells emulsified in complete Freund's adjuvant. As a control a group of 8 chickens was injected with PBS buffer emulsified in complete Freund's adjuvant. Before the immunization and 2, 4 and 6 weeks after immunization blood samples were taken. None of the control animals injected with PBS in complete Freund's adjuvant developed neutralizing antibodies against CAV (Table 1). Also chickens injected with lysates of $10^{6}$ or $10^{8}$ insect cells infected with recombinant VP2 or recombinant VP3 baculoviruses developed no neutralizing antibodies against CAV. Of the chickens injected with lysate of 106 infected recombinant VP1 baculovirus insect cells three chickens, and of the chickens infected with a dosage of $10^{8}$ infected cells two chickens developed low titers varying between 1:8 and 1:32. We conclude that the three recombinant CAV proteins, if infected separately into the chicken, induce no or only very slightly neutralizing antibodies against CAV.

Subsequently, we have studied whether the combination of the three recombinant CAV proteins is capable of inducing neutralizing antibodies in the chicken. To this end, Sf9 cells were infected simultaneously with the three recombinant CAV baculoviruses. Crude lysates of $10^{6}$ or $10^{8}$ of the infected cells, which therefore contained recombinant VP1+VP2+VP3, were prepared. Groups of eight chickens at an age of 6–8 weeks were injected with these lysates emulsified in complete Freund's adjuvant. As a control, a group of eight chickens was injected

TABLE 1

| Induction Of Neutralizing Antibodies After Immunization With Recombinant VP1 | | | | | |
|---|---|---|---|---|---|
| Chicken | Antigen | Neutralization Titer on Day | | | |
| No. | Dose§ | 0 | 14 | 28 | 42 |
| 1 | 0¶ | ≦4 | ≦4 | ≦4 | ≦4 |
| 2 | 0 | ≦4 | ≦4 | ≦4 | ≦4 |
| 3 | 0 | ≦4 | ≦4 | ≦4 | ≦4 |
| 4 | 0 | ≦4 | ≦4 | ≦4 | ≦4 |
| 5 | 0 | ≦4 | ≦4 | ≦4 | ≦4 |
| 6 | 0 | ≦4 | ≦4 | ≦4 | ≦4 |
| 7 | 0 | ≦4 | ≦4 | ≦4 | ≦4 |
| 8 | 0 | ≦4 | ≦4 | ≦4 | ≦4 |
| 9 | $10^6$ | ≦4 | ≦4 | ≦4 | ≦4 |
| 10 | $10^6$ | ≦4 | 8 | 32 | 8 |
| 11 | $10^6$ | ≦4 | ≦4 | ≦4 | ≦4 |
| 12 | $10^6$ | ≦4 | ≦4 | 8 | ≦4 |
| 13 | $10^6$ | ≦4 | ≦4 | ≦4 | ≦4 |
| 14 | $10^6$ | ≦4 | ≦4 | ≦4 | 16 |
| 15 | $10^6$ | ≦4 | ≦4 | ≦4 | ≦4 |
| 16 | $10^6$ | ≦4 | ≦4 | ≦4 | ≦4 |
| 17 | $10^8$ | ≦4 | ≦4 | ≦4 | ≦4 |
| 18 | $10^8$ | ≦4 | ≦4 | ≦4 | ≦4 |
| 19 | $10^8$ | ≦4 | ≦4 | 8 | 8 |
| 20 | $10^8$ | ≦4 | ≦4 | ≦4 | ≦4 |
| 21 | $10^8$ | ≦4 | ≦4 | ≦4 | ≦4 |
| 22 | $10^8$ | ≦4 | ≦4 | ≦4 | 8 |
| 23 | $10^8$ | ≦4 | ≦4 | ≦4 | ≦4 |
| 24 | $10^8$ | ≦4 | ≦4 | ≦4 | ≦4 |

§number of SF9 insect cells infected with recombinant baculovirus. Immunization was carried out with cell lysate.
¶animals injected with PBS instead of cell lysate

with PBS buffer emulsified in complete Freund's adjuvant. Five weeks after immunization, the eight chickens immunized with lysate of $10^6$ infected cells were all found to have neutralizing titers between 32 and 256, whereas seven of the eight animals immunized with $10^8$ cells had titers between 16 and 512 (Table 2A). Seven weeks after immunization all the animals of both groups were found to have developed a neutralizing titer against CAV. The group of chickens injected with PBS buffer was found to have developed no demonstrable neutralizing immune response against CAV.

Is it really necessary for the induction of neutralizing antibodies against CAV that the three CAV proteins are synthesized simultaneously in insect cells? To answer this question, Sf9 cells were infected separately with VP1, VP2 and VP3 recombinant baculoviruses. Then the crude cell lysates were combined, mixed with Freund's adjuvant and injected into a group of 8 chickens. As control preparations a crude lysate of Sf9 cells which all synthesized the 3 CAV proteins simultaneously and PBS buffer were used. Both preparations were emulsified in complete Freund's adjuvant and then injected into separate groups of each 8 chickens.

Sera of the group of chickens injected with crude lysates of Sf9 cells in which the 3 CAV proteins were synthesized separately found to contain no or only very few neutralizing antibodies against CAV. However, the animals of the control group injected with crude lysates of Sf9 cells which together synthesized the 3 CAV proteins were found, as expected, to have developed a neutralizing immune response. The animals infected with PBS buffer were found to be negative (Table 2B).

Neutralizing Antibodies in Eggs of Immunized Mother Animals

The above immunization experiments showed that 3 recombinant CAV proteins expressed together in Sf9 cells induced neutralizing antibodies against CAV. In a next experiment it was examined whether combinations of 2 CAV proteins were also capable of inducing neutralizing antibodies. Here the antibodies in the yolks of eggs of immunized mother animals were measured.

TABLE 2A

| Induction Of Neutralizing Antibodies With Immunization With Recombinant VP1, VP2 plus VP3 | | | | |
|---|---|---|---|---|
| Chicken | Antigen | Neutralization Titer on Day | | |
| No. | Dose§ | 15 | 35 | 42 |
| 1 | 0¶ | ≦4 | ≦4 | ≦4 |
| 2 | 0 | ≦4 | ≦4 | ≦4 |
| 3 | 0 | ≦4 | ≦4 | ≦4 |
| 4 | 0 | ≦4 | ≦4 | ≦4 |
| 5 | 0 | ≦4 | ≦4 | ≦4 |
| 6 | 0 | ≦4 | ≦4 | ≦4 |
| 7 | 0 | ≦4 | ≦4 | ≦4 |
| 8 | 0 | ≦4 | ≦4 | ≦4 |
| 9 | $10^6$ | 32 | 256 | 1024 |
| 10 | $10^6$ | 8 | 32 | 64 |
| 11 | $10^6$ | ≦4 | 64 | 256 |
| 12 | $10^6$ | 8 | 64 | 128 |
| 13 | $10^6$ | 4 | 64 | 128 |
| 14 | $10^6$ | ≦4 | 16 | 256 |
| 15 | $10^6$ | 8 | ≦128 | 256 |
| 16 | $10^6$ | 4 | ≦128 | 1024 |
| 17 | $10^8$ | 32 | ≦4 | 4 |
| 18 | $10^8$ | 8 | 512 | 256 |
| 19 | $10^8$ | 16 | 64 | 64 |
| 20 | $10^8$ | ≦4 | 64 | 256 |
| 21 | $10^8$ | ≦4 | 16 | 32 |
| 22 | $10^8$ | ≦4 | 32 | 128 |
| 23 | $10^8$ | 16 | 64 | 256 |
| 24 | $10^8$ | 4 | 64 | 128 |

§number of SF9 insect cells infected with recombinant baculovirus. Immunization was carried out with cell lysate.
¶animals injected with PBS instead of cell lysate

TABLE 2B

| Induction Of Neutralizing Antibodies After Immunization of Crude Lysates of Sf9 Cells Co-Infected with VP1, VP2, and VP3 Recombinant Baculovirus, or Mixture of Crude Lysates of Sf9 Cells Separately Infected with VP1, VP2, and VP3 Recombinant Baculovirus | | | | | |
|---|---|---|---|---|---|
| Chicken | | Neutralization Titer on Day | | | |
| No. | Immunization | 0 | 14 | 28 | 42 |
| 1042 | PBS | ≦2 | ≦2 | ≦2 | ≦2 |
| 1044 | PBS | ≦2 | ≦2 | ≦2 | |
| 1046 | PBS | ≦2 | ≦2 | ≦2 | |
| 1048 | PBS | ≦2 | ≦2 | ≦2 | |
| 1051 | PBS | ≦2 | ≦2 | ≦2 | |
| 1053 | PBS | ≦2 | ≦2 | ≦2 | |
| 1056 | PBS | ≦2 | ≦2 | ≦2 | |
| 1084 | PBS | ≦2 | ≦2 | ≦2 | |
| 1058 | #together | ≦2 | ≦2 | 128 | 256 |
| 1060 | together | ≦2 | 16 | 512 | 512 |
| 1062 | together | ≦2 | ≦2 | 64 | 128 |
| 1064 | together | ≦2 | 16 | 128 | 256 |
| 1066 | together | ≦2 | 4 | 64 | 64 |
| 1068 | together | ≦2 | 16 | 256 | N.D. |
| 1070 | together | ≦2 | 16 | 128 | 512 |
| 1072 | together | ≦2 | 16 | 256 | 512 |
| 1074 | apart& | ≦2 | ≦2 | 8 | 8 |
| 1078 | apart | ≦2 | 2 | ≦2 | ≦2 |
| 1081 | apart | ≦2 | 2 | ≦2 | ≦2 |
| 1083 | apart | ≦2 | ≦2 | ≦2 | ≦2 |
| 1085 | apart | ≦2 | ≦2 | 2 | 8 |
| 1087 | apart | ≦2 | ≦2 | ≦2 | |
| 1089 | apart | ≦2 | ≦2 | ≦2 | |
| 1091 | apart | ≦2 | ≦2 | ≦2 | |

TABLE 2B-continued

Induction Of Neutralizing Antibodies After Immunization of Crude Lysates of Sf9 Cells Co-Infected with VP1, VP2, and VP3 Recombinant Baculovirus, or Mixture of Crude Lysates of Sf9 Cells Separately Infected with VP1, VP2, and VP3 Recombinant Baculovirus

| Chicken | | Neutralization Titer on Day | | | |
|---|---|---|---|---|---|
| No. | Immunization | 0 | 14 | 28 | 42 |

#immunization with crude lysates of Sf9 cells co-infected with VP1, VP2, and VP3-recombinant baculovirus.
&Immunization with mixtures of crude lysates of Sf9 cells separately infected with VP1, VP2, and VP3 recombinant baculovirus.

Four groups of each 16 hens at an age of 33 weeks were injected with crude lysates of 2×107 Sf9 cells, which were simultaneously infected with VP1, VP2, and VP3 recombinant baculoviruses; or with VP1 and VP2; or with VP1 and VP3; or with VP2 and VP3 recombinant baculoviruses. The cell lysates were emulsified in an equal volume of complete Freund's adjuvant. As a control a group of 16 hens was injected with PBS buffer in complete Freund's adjuvant. Yolk material of eggs of hens injected with these lysates or with PBS buffer was extracted with chloroform and analyzed for the presence of neutralizing antibodies.

The preparations containing either VP1+VP2+VP3 or VP1+VP2 induced in most animals neutralizing antibodies clearly demonstrable in their eggs (Table 3). The eggs of chickens injected with preparations containing either VP1+VP3 or VP2+VP3 were found to have no clear neutralizing antibody titer in the yolks. Only the yolks of eggs of one of the examined chickens were found to contain low titers of neutralizing antibodies. The eggs of the control group of 16 chickens injected with PBS buffer were found to contain no neutralizing antibodies.

The data from the above-mentioned experiments with recombinant CAV proteins show that VP1+VP2 together are necessary and sufficient for the induction of neutralizing antibodies against CAV infections. However, a minor amount VP3 in the VP1+VP2 preparations cannot be ruled out.

## Example 3

### Production and Characterization of Monoclonal Antibodies Specifically Directed Against CAV Proteins

For the production of monoclonal antibodies against CAV, mice were injected with crude lysates of Sf9 cells co-infected with VP1, VP2 and VP3 recombinant baculoviruses. In total, 9 different hybridoma cell lines producing monoclonal antibodies against CAV antigens were obtained.

The monoclonal antibody CVI-CAV-85.1 was obtained by injecting mice intraperitoneally with CAV injected MDCC-MSB1 cells with incomplete Freund's adjuvant. Finally, spleen cells of the immunized mice were fused with P3X63-Ag8.653 myeloma cells (Noteborn et al., (1991) J. Virology 65:3131–3139).

The other monoclonal antibodies directed against CAV antigens were obtained by injecting crude extracts of Sf9 cells infected with the three CAV recombinant

TABLE 3

Neutralizing Antibodies in Egg Yolks of Chicks After Immunization With a Combination of Recombinant VP1, VP2, and VP3

| Animal No. | Immunization | No. of Eggs | Average Titer |
|---|---|---|---|
| 1194 | PBS | 1 | ≦4 |
| 1211 | PBS | 3 | ≦4 |
| 1231 | PBS | 3 | ≦4 |
| 1132 | PBS | 4 | ≦4 |
| 1233 | PBS | 4 | ≦4 |
| 1251 | PBS | 4 | ≦4 |
| 1254 | PBS | 2 | ≦4 |
| 1195 | VP1 + VP2 + VP3 | 5 | ≦4 |
| 1196 | VP1 + VP2 + VP3 | 3 | 16 |
| 1197 | VP1 + VP2 + VP3 | 1 | 4 |
| 1199 | VP1 + VP2 + VP3 | 2 | 64 |
| 1203 | VP1 + VP2 + VP3 | 4 | 22.6 |
| 1205 | VP1 + VP2 + VP3 | 1 | 32 |
| 1206 | VP1 + VP2 + VP3 | 3 | 128 |
| 1207 | VP1 + VP2 + VP3 | 3 | 32 |
| 1208 | VP1 + VP2 + VP3 | 3 | 12.6 |
| 1210 | VP1 + VP2 + VP3 | 4 | 32 |
| 1216 | VP1 + VP2 | 3 | 64 |
| 1219 | VP1 + VP2 | 4 | 45.2 |
| 1220 | VP1 + VP2 | 3 | 32 |
| 1223 | VP1 + VP2 | 3 | 50.8 |
| 1224 | VP1 + VP2 | 4 | 76 |
| 1226 | VP1 + VP2 | 3 | 40.4 |
| 1227 | VP1 + VP2 | 4 | 19 |
| 1228 | VP1 + VP2 | 2 | 8 |
| 1229 | VP1 + VP2 | 3 | 4 |
| 1230 | VP1 + VP2 | 3 | 32 |
| 1235 | VP1 + VP3 | 3 | ≦4 |
| 1238 | VP1 + VP3 | 3 | ≦4 |
| 1239 | VP1 + VP3 | 1 | ≦4 |
| 1245 | VP1 + VP3 | 3 | ≦4 |
| 1248 | VP1 + VP3 | 2 | ≦4 |
| 1249 | VP1 + VP3 | 3 | ≦4 |
| 1255 | VP2 + VP3 | 3 | ≦4 |
| 1258 | VP2 + VP3 | 5 | ≦4 |
| 1259 | VP2 + VP3 | 4 | ≦4 |
| 1260 | VP2 + VP3 | 4 | ≦4 |
| 1261 | VP2 + VP3 | 4 | ≦4 |
| 1263 | VP2 + VP3 | 4 | ≦4 |
| 1264 | VP2 + VP3 | 4 | 9.6 |
| 1265 | VP2 + VP3 | 3 | ≦4 |
| 1266 | VP2 + VP3 | 3 | ≦4 |
| 1267 | VP2 + VP3 | 2 | ≦4 |
| 1268 | VP2 + VP3 | 3 | ≦4 |
| 1269 | VP2 + VP3 | 3 | ≦4 |
| 1270 | VP2 + VP3 | 4 | ≦4 |

baculoviruses into the spleen of 4 BALB/c mice. The sera of the immunized mice were tested for 7 weeks after immunization for neutralizing antibodies against CAV. The spleen cells of the immunized mice were fused with P3X63-Ag8.653 myeloma cells. Antibodies directed against CAV antigens were tested by different ways: aserum neutralization test; ELISAs based on purified CAV and on crude lysates of Sf9 cells infected with CAV recombinant baculovirus; immunofluorescence tests on CAV infected MDCC-MSB1 or on Sf9 cells infected with CAV recombinant baculovirus; Western blots of crude lysates of Sf9 cells infected with CAV recombinant baculovirus, and immunoperoxidase staining on thymus coupes of CAV infected chickens.

Western blots with CAV antigens produced with the baculovirus expression system showed that the monoclonal antibodies 111.1, 111.2, 111.4, 112.1, 112.2, 120.1 and 120.2 are strongly directed against VP2 and the monoclonal antibodies 111.3 and 120.3 strongly against VP3. The monoclonal antibodies which strongly react with VP2 all show a weak cross reaction with VP3. Conversely, the monoclonal antibodies directed against VP3 show a weak cross reaction with VP2.

## Example 4

### Analysis of Antibodies Against CAV Antigens

In vitro neutralization test

The sera of chickens and mice infected with crude Sf9 cell lysates or PBS buffer were diluted 1:2 or 1:4 and then a two-fold dilution series was made. The diluted sera were incubated for 1 hour with $10^4$-$10^5$ $TCID_{50}$ CAV-Cux-1 (Von Bülow et al., (1983) J. Vet. Med. B 30:742–750; Von Bülow, (1985) J. Vet. Medicine B 32:679–693. Approximately one hundred thousand cells of the T cell line MDCC-MSB1 transformed by Marek's disease virus were infected with this mixture of diluted sera and virus. As controls MDCC-MSB1 cells were infected with CAV which was neutralized with a positive CAV antiserum and a negative serum originating from specific pathogen free chickens.

The serum neutralization test showed that none of the monoclonal antibodies obtained had a neutralizing activity against CAV, in spite of the fact that the sera of the immunized mice used for preparing the hybridomas did have a neutralizing activity against CAV.

In a pepscan analysis (Geysen et al., (1984) Proc. Nat'l. Acad. Sci. (USA) 82:1978–1982) the epitope of the monoclonal antibody 111.2 was localized in the middle of VP2 (FIG. **6**). The monoclonal antibody 111.3 was found to be directed against an epitope at the N terminal end of VP3 (FIG. **7**), namely beside the VP3 epitope recognized by the monoclonal antibodies CVI-CAV-85. 1 (FIG. **5**).

CAV challenge experiments

Maternal antibodies protect young chicks against clinical symptoms caused by a CAV infection. We have studied which group(s) of chickens immunized with specific recombinant CAV proteins became offspring protected against CAV challenge.

Groups of between 23 and 35 day-old offspring were challenged with high doses of CAV. Six days after infection, virus was isolated and the animals evaluated for clinical symptoms characteristic of CAV: atrophy of the thymus, decreased hematocrit and increased mortality. Five animals which were subjected to section and which had mother animals injected with PBS buffer, were all found to have a macroscopically visibly reduced thymus. In case of offspring of mother animals injected with recombinant VP2+VP3, four of the five animals had a small thymus. However, the five offspring, subjected to section, of mother animals injected with the three recombinant CAV proteins together were all found to have a normal thymus. In the group of offspring of mother animals treated with VP1+VP2 only one of the five animals examined was found to have a reduced thymus (Table 4).

Fourteen days after infection, again five animals per group were subjected to section. All offspring of mother animals immunized with recombinant VP2+VP3 or PBS buffer suffered from thymus atrophy. The examined offspring of the group of animals injected with the three recombinant CAV proteins together were all found to have normal thymuses. Only one of the five examined chicks of the animals injected with recombinant VP1+VP2 was found to have a reduced thymus (Table 4). An independent experiment showed that offspring of mother animals injected with recombinant VP1 and VP3 had reduced thymuses, as described for the offspring of mother animals injected with recombinant VP2 and VP3.

TABLE 4

Section Findings after CAV Challenge in Offspring of Mother Animals Immunized with Recombinant CAV Products

| Group 1 VP1 + VP2 + VP3 | Group 2 VP1 + VP2 | Group 3 VP2 + VP3 | Group 4 PBS |
|---|---|---|---|
| | | day 6 after infection | |
| 0/5# | 1/5 | 4/5 | 5/5 |
| | | day 14 after infection | |
| 0/5 | 1/5 | 5/5 | 5/5 |
| | | more than 14 days after infection | |
| 1/3 (ND: (2/2)& | 0/0 | 13/14 (ND: 1/14) | 6/6 |

#Number of animals with small thymus.
&No section performed because of a specific mortality.

Fourteen days after infection the hematocrit of all CAV infected offspring was determined. A hematocrit of 27% was selected as the limit for anemia. The offspring of the mother animals injected with PBS buffer were all found to have a strongly reduced hematocrit, with values varying between 7 and 19% (Table 5). Offspring of the mother animals injected with recombinant VP2+VP3 have a slightly higher hematocrit on average. In these groups only a single animal had a hematocrit higher than 27. An independent experiment showed that also offspring of mother animals injected with recombinant VP1 and VP3 had a reduced hematocrit. Of the 35 examined offspring of the animals injected with preparations containing VP1, VP2 and VP3, only one animal had a deviating hematocrit, whereas in the VP1+VP2 group, two of the 29 examined animals had a hematocrit below 27%.

TABLE 5

Hematocrit values in offspring of mother animals immunized with combinations of recombinant-CAV baculo products

| VP1 + VP2 + VP3 | VP1 + VP2 | VP2 + VP3 | PBS |
|---|---|---|---|
| 37¶ | 29 | 14 | 18 |
| 30 | 31 | 20 | 11 |
| 33 | 34 | 13 | 16 |
| 33 | 30 | 28 | 15 |
| 34 | 35 | 25 | 19 |
| 28 | 34 | 8 | 13 |
| 34 | 22 | 28 | 9 |
| 32 | 34 | 12 | 11 |
| 29 | 36 | 6 | 17 |
| 30 | 37 | 7 | 14 |
| 29 | 32 | 18 | 10 |
| 36 | 30 | 16 | 17 |
| 31 | 25 | 19 | 18 |
| 32 | 36 | 14 | 7 |
| 28 | 34 | 29 | 8 |
| 32 | 33 | 13 | 10 |
| 33 | 32 | 8 | 8 |
| 31 | 36 | 31 | 12 |
| 37 | 34 | 14 | 14 |
| 32 | 28 | 25 | 9 |
| 38 | 32 | 19 | 11 |
| 30 | 35 | 15 | 8 |
| 33 | 36 | 7 | 12 |
| 23 | | 17 | 17 |
| 38 | | 14 | 12 |
| 37 | | 9 | 13 |
| 31 | | 18 | |

TABLE 5-continued

Hematocrit values in offspring of mother animals immunized with combinations of recombinant-CAV baculo products

| | VP1 + VP2 + VP3 | VP1 + VP2 | VP2 + VP3 | PBS |
|---|---|---|---|---|
| | 32 | | 8 | |
| | 29 | | 12 | |
| | 32 | | 14 | |
| | 32 | | | |
| | 31 | | | |
| | 32 | | | |
| | 34 | | | |
| | 32 | | | |
| average: | 32.1 | 32.4 | 16.0 | 12.7 |
| stand. dev. | 3.09 | 3.66 | 6.98 | 3.52 |
| max–min. | 23–38 | 22–37 | 6–28 | 3, 52 |
| number | n = 35 | n = 23 | n = 30 | n = 26 |

¶Hematocrit in individual animals.

A high mortality rate was observed with offspring of mother animals injected with recombinant VP2 and VP3, 50.9% and with PBS, 48.3%. In the group of offspring of mother animals injected with recombinant VP1+VP2+VP3 the mortality is 9% and with VP1+VP2 15.4%. However, most of the animals died within five days after challenge. The mortality caused by a CAV infection is generally somewhat later. For this reason we have distinguished in Table 6 between mortality before day 14 and after day 14 after challenge. The mortality before day 14 is often aspecific, inter alia as a result of injection. The mortality after day 14 is in the group of animals with maternal antibodies against VP1+VP2−VP3, 7%; against VP1+VP2, 0%, VP2+VP3, 27.4% and in the control group 20.7%. In the VP2+VP3 group, 8 animals died after taking blood samples for determining the hematocrit as a result of the poor condition of the chicks, most probably caused by the anemia. In the PBS group, two animals died during blood taking. All these animals had a clearly reduced thymus.

TABLE 6

Mortality After CAV Challenge in Offspring of Mother Animals Immunized with Recombinant CAV Products

| Group 1 VP1 + V#+ VP3 | Group 2 VP1 + VP2 | Group 3 V#+ VP3 | Group 4 PBS |
|---|---|---|---|
| | | before day 14 after injection | |
| 1/43 (2%) | 7/39 (15.4%) | 12/51 (23.5%) | 8/29 (27.6%) |
| | | after day 14 after injection | |
| 3/43 (7%) | 0/39 | 14/51 (27.4%) | 6/29 (20/7%) |

The viremia in the CAV infected offspring was examined by carrying out a virus isolation on blood cells. Heparin blood samples of five animals per group were taken on 6 and 14 days after challenge. The offspring of mother animals injected with VP2+VP3 or PBS, and which had practically no protection against CAV infections, were found to contain relatively high virus titers 6 and 14 days after infection. Six days after infection the offspring of animals injected with VP1+VP2+VP3 or VP1+VP2 were found to contain a clearly lower virus titer than the above-mentioned offspring. Fourteen days after infection only the group of offspring of animals injected with VP1+VP2+VP3 had a clearly lower virus titer than the other three groups.

The results of the induction of neutralizing antibodies in mother animals show that the recombinant CAV proteins VP1 and VP2 are very important for the induction of a neutralizing immune response. The infection experiments show that the recombinant CAV protein VP3 gives a supplementary protection in addition to the effect obtained by VP1+VP2. Fertilized eggs of the five groups of immunized hens were hatched. The chicks were injected intramuscularly on day 1 with $10^{5.5}$ $TCID_{50}$ CAV-Cux-1. On 6 and on 14 days after infection 5 chickens per group were subjected to section. The thymus was analyzed macroscopically and immunohistologically. Also, heparin blood was taken, and the blood cells were tested in a virus reisolation assay. Fourteen days after infection heparin blood was collected from all animals to determine the hematocrit.

## Example 5

### Immunohistology and Immunofluorescence

Frozen coupes of thymus and bone marrow were made and used for immunoperoxidase staining with CAV-specific monoclonal antibodies, as described by Jeurissen et al. (1988) Vet. Immunol. Immunopathol. 19:225–238). Cells were fixed with 80% acetone and used for immunofluorescence tests with CAV-specific monoclonal antibodies and goat anti-mouse IgG conjugated with fluorescein isothiocyanate (Noteborn et al. (1990) Immunofluorescence showed that monoclonal antibodies directed against VP2 and VP3 recognize specific structures in CAV infected MDCC-MSB1 cells. None of the monoclonal antibodies directed against CAV antigens reacted with uninfected MDCC-MSB1 cells. The VP2-specific monoclonal antibodies recognize other structures than VP3 specific monoclonal antibodies in CAV infected cells.

Detection of CAV in blood samples

Blood samples of CAV infected chicks were washed thrice with PBS and taken up in 1 ml. Twenty microliters of the cell suspension obtained were added to $10^5$ MDCC-MSB1 cells. The MDCC-MSB1 cells were 10 times diluted every 4–5 days, transferred to fresh culture medium, until a CAV-specific cytopathogenic effect became visible. If after 10 passages no cytopathogenic effect could be observed yet, then the virus isolation was considered to be negative. The number of times of passage is a measure for the amount of infectious CAV present in the blood of the infected chicks.

## Example 6

### Simultaneous Expression of Recombinant VP1 and VP2

Construction of a Recombinant-VP1/VP2 Transfer Vector

The coding sequences for the CAV proteins VP1 and VP2 were cloned into the baculovirus transfer vector pAcUW51 (cat. no: 21205P), which was commercially obtained from PharMingen, San Diego, USA. This vector is shown in FIG. **13** and contains the polyhedrin flanking region, within their midst the baculovirus polyhedrin promoter and the p10 promoter and for both transcription units, the required 3'-non-coding transcriptional sequences including the polyadenylation signals. The transfer vector contains prokaryotic sequences for multiplication in bacteria.

The plasmid pET-16b-VP2 contains CAV DNA sequences of positions 380 to 1512. This CAV DNA fragment contains the coding region for VP2 flanked by 484 bp 3'-non-coding CAV DNA sequences. 106 bp downstream of the start codon for VP2 the start codon for VP3 is found in another reading frame. The plasmid pET-16b-VP2 was treated with the restriction enzymes NdeI and NheI, and the sticky ends were filled by means of Klenow polymerase. A 0.8 kb CAV DNA fragment was isolated. The plasmid pAcUW51 was linearized with BamH1, the stick ends filled by means of Klenow polymerase and finally treated with alkaline phosphatase (CIP). The 0.8 kB CAV DNA fragment was ligated at the linearized pAcUW51 DNA. The orientation of VP2 in pAcUW51 was determined by restriction enzyme analysis. This construct was called pUW-VP2.

The plasmid pET-16b-VP1 contains CAV DNA sequences of positions 853 to 2319. The CAV DNA insertion contains the complete coding region for the protein VP1 flanked by 117 bp 3'-non-coding CAV DNA sequences. The plasmid pET-16b-VP1 was treated with the restriction enzymes NdeI and EcoRI, and the sticky ends were filled by means of Klenow polymerase. A 1.45 kb CAV DNA fragment was isolated. The plasmid pUW-VP2 was linearized by EcoRI, the sticky ends filled by means of Klenow polymerase and finally treated by CIP. The 1.45 kb CAV DNA fragment was ligated at the linearized pUW-VP2. The orientation of VP1 opposite of the p10 promoter unit was determined by restriction-enzyme analysis, and the final construct pAcVP1/VP2 is shown in FIG. **13**.

Construction of recombinant-VP1/VP2 baculovirus

Recombinant transfer vector pAcVP1/VP2 DNA was transfected with linearized recombinant baculovirus AcRP23-lacZ DNA, in sf9 cells. After homologous recombination baculoviruses were obtained, which had incorporated in the polyhedrin unit instead of the lacZ the two CAV proteins VP1 and VP2 under regulation of the promoter of the p10 or polyhedrin gene, respectively. In the first instance, the recombinant CAV viruses were characterized for the absence of β-galactosidase activity in plaques of baculovirus-infected insect cells. Further the integration of CAV DNA sequences in the baculovirus genome was determined by means of a CAV-specific DNA probe in a hybridization experiment.

Expression of the CAV proteins VP1 and VP2 in sf9 cells

The simultaneous expression of the CAV proteins VP1 and VP2 in sf9 cells infected with recombinant-VP1/VP2 baculovirus was analyzed by Coomassie-brilliant blue staining and protein labeling with Promix (ICN, USA) or $^{3}$H-leucine (Amersham, UK) and PAA-SDS gel electrophoresis.

As described above, lysates of insect cells infected with recombinant-VP1 baculovirus contained a CAV-specific protein of 52 kD and expression of VP2 by recombinant-VP2 baculovirus in infected insect cells produced a major specific product of 30 kDa. Infection of insect cells with recombinant-VP1/VP2 baculovirus resulted in the synthesis of both the CAV-specific proteins of 52 kD and 30 kD. Both CAV-specific products could be detected as either a radioactively labeled protein band or a Coomassie-brilliant blue stained protein band. The latter result indicates that both products are produced in relatively high levels in recombinant-VP1/VP2-baculovirus-infected insect cells. sf9 cells infected with a recombinant-lacZ baculovirus did not contain these CAV-specific proteins.

As expected, we have obtained evidence that inoculation in hens with crude lysates of recombinant-VP1/VP2-infected sf9 cells induces neutralizing antibodies directed against CAV.

The production and characterization of neutralizing monoclonal antibodies against CAV

Two types of CAV-specific monoclonal antibodies are described above. One type is directed against VP2, while the other is directed against VP3. Neither of these types of monoclonal antibodies reveal a CAV-specific neutralizing activity. Even more important was that none of these monoclonal antibodies was directed against VP1. We assumed that a neutralizing monoclonal antibody might be directed against VP1, for the capsids contain mainly VP1 (Todd et al., (1990) G. Gen Virol. 71:819–823). Below, we describe the production of neutralizing antibodies against CAV.

For the production of neutralizing monoclonal antibodies against CAV, mice were injected with purified CAV particles. The supernatant of a 1 liter culture of CAV-infected MDCC-MSB1 cells was concentrated forty fold by means of a MILLITAN 300-kDa filter (Millipore, USA). The supernatant was dialyzed against 10 mM Tris(pH 8.7)-1 mM EDTA (TE) buffer. Subsequently, sodium dodecyl sulphate (SDS) (0.5% final concentration) was added to the CAV-capsid suspension and incubated for 30 minutes at 37° C. Finally, the CAV capsids were pelleted on a 30% sucrose cushion. The pellet containing the CAV capsids was resuspended in 1 ml TE buffer. Mice were twice injected with 100 ul CAV-capsid suspension.

As a first screening for (neutralizing) monoclonal antibodies against CAV, microtitre wells were coated with recombinant-VP1/VP2-baculovirus infected insect cells, which co-synthesized both VP1 and VP2. CAV-specific antisera with a high neutralizing titer reacted at a dilution of 1:1000 specifically with the recombinant VP1 and/or VP2 products (see below). Several different hybridoma cell lines producing monoclonal antibodies, which specifically reacted with recombinant VP1/VP2 products, were obtained.

A CAV-specific serum neutralization test, carried out as described above, showed that three of these monoclonal antibodies obtained, had a neutralizing activity against CAV. These three CAV-specific neutralizing monoclonal antibodies were called 132.1; 132.2 and 132.3. Immunofluorescence showed that the three neutralizing monoclonal antibodies 132.1, 132.2 and 132.3 recognize specific structures in CAV-infected MDCC-MSB1 cells. None of these monoclonal antibodies reacted with uninfected MDCC-MSB1 cells.

Electron-microscopic analysis was carried out with purified CAV particles incubated with neutralizing antibodies against CAV (132.1) or with monoclonal antibodies 111.1 (against VP2) or 111.3 (against VP3). The various monoclonal antibodies were detected by immunogold labeling. Only the neutralizing monoclonal antibodies 132.1 were found to bind to CAV particles. Binding of the monoclonal antibody 132.1 to a CAV particle resulted in its lysis. Furthermore, CAV capsids, which were lysed due to incubation with the neutralizing monoclonal antibody 132.1, showed no binding with monoclonal antibodies directed against VP2 or VP3.

These results reveal the mechanism by which the neutralizing monoclonal antibodies act: they cause the lysis of the virus capsids, by doing so causing non-infectious particles. Furthermore, these data suggest that purified CAV particles contain (almost) only VP1.

Pepscan analysis (Gheysen et al., (1984) Proc. Nat'l Acad Sci. (USA) (82:178–182) revealed that none of the three neutralizing monoclonal antibodies reacted significantly with one of the 12-mers derived from VP1 or VP2. For the sake of brevity only the data obtained with monoclonal antibody 132.1 are shown for VP1 in FIG. **14**, and for VP2 in FIG. **15**. These results indicate that the neutralizing monoclonal antibodies are directed against a conformational epitope. These data were confirmed by the following experiments. Purified CAV particles, dotted on a nylon filter under native conditions, still could react with the neutralizing monoclonal antibody 132.1. However, after boiling in the presence of SDS, the CAV capsid proteins did not bind to monoclonal antibody 132.1.

Immunoprecipitation experiments, carried under native conditions, as described by Noteborn et al., In: Virus Diseases of Poultry-New and Evolving Pathogens, (1994) 195–212, with partially purified CAV particles and monoclonal antibody 132.1, 132.2 or 132.3 showed that a protein of about 50 kDa was precipitated by these monoclonal antibodies. These results indicate that the neutralizing monoclonal antibodies are directed against VP1.

The role of VP2 for the formation of the neutralizing epitope of VP1

As reported above, simultaneous synthesis and not simply mixing of recombinant CAV proteins VP1 and VP2 is required to obtain a neutralizing and protective immune response suggesting that VP2 is a non-structural protein that at some stage of infection is required for virus assembly and/or the correct conformation of VP1, which result(s) in the formation of the neutralizing epitope(s). One explanation of the requirement of VP2 might be that it acts as a scaffold protein that is necessary during the assembly of the virion but absent in the final product. Examples of scaffold proteins are the IVa2 and 39kDa proteins of adenovirus (D'Halluin et al., (1978) J. Virol. 26:357–363; Persson et al., (1979) Virology 93:198–208. These proteins act as scaffolds for the formation of the so-called light capsid, but are removed in the next step. VP2 might function in a similar way during the formation of CAV virions. However, at this stage, we cannot entirely exclude that (very) small amounts of VP2 that remained undetected in electroblots of purified CAV preparations or in electron microscopic photographs of lysed CAV particles incubated with immunogold-labeled VP2-specific monoclonal antibodies, as described above, associate with VP1 and form conformational neutralizing epitopes. Recently, evidence for the presence of VP2 in gradient-purified CAV was reported (Buchholdz, (1994) Characterization of the Chicken Anemia Virus (CAV) with help from monoclonal antibodies. Dissertation Free University of Berlin, 1994, Journal no 1738, Berlin, Germany.

In the following experiments, evidence is provided that the neutralizing epitope of VP1 is only (optimally) present, when VP2 is simultaneously synthesized. Insect cells were infected with recombinant-CAV baculoviruses expressing VP1, VP2 (PCT/NL94/00168) or both VP1 plus VP2. The infected sf9 cells were harvested 3 or 4 days after infection and fixed with 80% acetone and used for immunofluorescence tests with the CAV-specific neutralizing monoclonal antibody 132.1 and goat anti-mouse IgG conjugated with fluorescein isothiocyanate (Noteborn et al., (1990). The cells containing only the CAV-specific protein VP2 did not react at all with the monoclonal antibody 132.1. Cells containing only VP1 revealed a very poor immunofluorescence signal after incubation with monoclonal antibody 132.1. However, insect cells infected with recombinant-VP1/VP2 baculovirus expressing both VP1 and VP2 bound very strongly to the neutralizing monoclonal 132.1. PAA-SDS gel electrophoresis of in parallel radioactive-labeled lysates of insect cells expressing VP1, VP2 or VP1 plus VP2, revealed that VP1 is expressed at the same level when expressed only or simultaneously with VP2.

In conclusion, the neutralizing epitope of VP1 is only formed when VP2 is present. This implies that VP1 and VP2 associate with each other during a short time period. By means of immunoprecipitation under very mild conditions, we have examined whether VP1 could associate with VP2. sf9 insect cells were infected with recombinant baculoviruses, which synthesized VP1, VP2, or VP1 plus VP2. Two days after infection, the cells were incubated with Promix label (ICN, USA) and four hours later, the cells were lysed in E1A buffer (50 mM Tris (pH 7.5), 0.1% Triton-X-100, 250 mM NaCl, 50 mM NaF, and 5 mM EDTA) and incubated with monoclonal antibody 111.1 directed against VP2 for two hours at 4° C., washed with E1A buffer and separated on a PAA-SDS gel. The results clearly reveal that monoclonal antibody 111.1 precipates VP2 when VP2 is synthesized alone or in the presence of VP1. In the case that VP1 was expressed in addition to VP2, VP1 co-precipitated to a small extent with VP2. The monoclonal antibody 111.1 did not detectably precipitate VP1, when VP1 was synthesized in the absence of VP2. These data indicate that VP1 and VP2 are (to a relatively small amount) associated to each other. During this association event, VP1 might obtain its conformation resulting in the neutralizing epitope.

Basis for the development of vaccines against CAV infections

The above presented results together with those described in PCT/NL94/00168 show that for the induction of neutralizing antibodies against CAV, VP1 is needed to have a specific conformation. In a baculovirus expression system, this correct VP1 confirmation is only possible, when VP1 plus VP2 or VP1 plus VP2 plus VP3 are simultaneously synthesized.

The recombinant CAV products, VP1 plus VP2 or VP1 plus VP2 plus VP3, which will be used for vaccination of laying-hens, can be synthesized by means of the baculovirus system. The CAV proteins can also be synthesized by means of other expression systems, such as yeast cells, via (retro)-viral infection or gene amplification (CHO-dhfr system) in mammalian cell systems.

In principle, the expression of fragments of VP1 (in combination with VP2 or VP2 and VP3) may be sufficient for the induction of a protective immune response. The fact that 12-mers of VP1 can not react with neutralizing antibodies against CAV indicates that larger VP1 fragments are needed for getting the correct VP1 conformation to form the neutralizing epitope. However, one should take into account that minor amino-acid mutations or a few amino-acid deletions might not influence the formation of the neutralizing epitope of VP1.

That fact that two or three proteins encoded by the CAV open reading frames can induce a protective immune response is also applicable to the development of living virus vectors. The coding sequences for VP1 plus VP2 or VP1 plus VP2 plus VP3 are then cloned into living virus vectors.

It is also possible that one of the CAV proteins, VP1, VP2 or VP3, separately, but then within the context of a living virus vector, is also suitable for the induction of a protective immune response against CAV infections. The expression of fragments of one of the above-mentioned CAV proteins by living virus vectors may be sufficient for the induction of a protective immune response.

The fact that VP3 causes apoptosis in, i.e., chicken mononuclear cells (FCT/NL94/00168) makes it preferable to prepare living virus vectors not expressing VP3. The replication of i.e., Marek virus might be negatively influenced by VP3-induced apoptosis. Alternatively, one can construct living virus vectors expressing VP1, VP2 and a truncated VP3 lacking the C-terminal 11 amino acids resulting in a strong induction of apoptosis by VP3.

Enzyme-linked immunosorbens assay (ELISA) based on a neutralizing antibody against CAV

A complex-trapping-blocking (CTB)-ELISA has been constructed using enriched CAV particles derived from CAV-infected MDCC-MSB-1 cells or recombinant VP1/VP2 proteins synthesized by means of the above described baculovirus system.

Microtiter wells (Greiner, FRG) were coated with the CAV-specific neutralizing monoclonal antibody 132.1, which was 1:10,000 diluted in 50 mM sodiumbicarbonate pH 9.6. Wash the wells three times with tap water containing 0.05% Tween 80. Saturate the wells with 100 μl phosphate-buffered saline containing 4% horse serum, 51 gram/liter NaCl, and 0.05% Tween 80. Nxt, 50 μl of non-diluted chicken serum and 50 μl of thirty times concentrated supernatant containing CAV particles, or 50 μl of a lysate of insect cells containing recombinant VP1 and VP2 proteins, were mixed, added per well and incubated for 1 hour at 37° C. The wells were washed three times with tap water containing 0.05% Tween 80. 100 μl of a standard solution of tetramethylbenzidine, sodiumacetate and hydrogenperoxidase was added to the wells and incubated for 10 minutes at room temperature. The reactions were blocked with 10% $H_2SO_4$. The various wells were examined at 450 nm, as standard.

Serum from CAV-infected chickens contains antibodies which will block all epitopes on the CAV capsids or recombinant VP1/VP2. This means that the CAV capsid or recombinant VP1/VP2 will not bind to the coated monoclonal antibody 132.1. Negative serum, however, will allow binding of CAV capsids or recombinant VP1/VP2 to the coated 132.1. A signal smaller than 0.5 of the signal detected with a negative control serum will be examined as positive.

The detection level of our CTB-ELISA are titers of 24 to 25 as determined in a serum neutralization test, which is very sensitive. More than 400 sera were analyzed. Comparison to the serum neutralization test revealed that 96.5% of the positive sera within the serum neutralization test were positive within the CTB-ELISA, and 98.3% of the negative sera within the serum neutralization test were negative within the CTB-ELISA.

## Example 7

## Expression of VP3 in Human Tumor Cells Induces Apoptosis

For the expression of VP3 in human cells the expression vectors pRSV-VP3 (FIG. **8**A) and pCMV-VP3 were used. The coding sequences for VP3 were cloned into the expression vector pCMV-neo containing the strong promoter of the cytomegalovirus (CMV) immediate early gene (Boshart et al., 1985). The 0.46 BamH I fragment with CAV DNA sequences of positions 427–868 (Noteborn et al., 1991) were isolated from plasmid pAc-VP3 (FIG. **4**). The vector pCMV-neo was linearized with BamH I, treated with CIP; and a 7.5 kb fragment was isolated. The 0.46 BamH I DNA fragment was ligated at the 7.5 BamH I DNA fragment. The right orientation of the VP3-coding sequence with respect to the CMV promoter in the final construct pCMV-VP3 was determined by means of restriction enzyme analysis (FIG. **10**).

For the expression of truncated VP3 in human cells the 0.46 kb Xho I-Sal I fragment of plasmid pRSV-tr coding for truncated VP3 (FIG. **8**A) was provided with blunt ends by treatment with Klenow polymerase and isolated. The vector pCMV-neo was linearized with BamH I, provided with blunt end and dephosphorylated by treatment with CIP. The 0.46 kb blunt end DNA fragment was ligated at the 7.5 blunt end DNA fragment. The construct pCMV-tr contains the coding sequences for truncated VP3 under regulation of the CMV promoter (FIG. **10**).

In the first instance, VP3 was expressed in the 3 human hematopoietic tumor cell lines KG-1, DOHH-2, K562, and in an immortalized cell line, Jobo-0. The cell lines KG-1 and K562 have been derived from different patients with human myeloid leukemia (Koeffler and Golde, 1980) and DOHH-2 from a patient with a follicular B-lymphoma (Landegent et al, results not published). Jobo-0 cells were immortalized with the Epstein Barr Virus (Landegent, results not published). The 4 human cell lines were transfected with DNA of pRSV-VP3 (KG-1) or with DNA of pCMV-VP3 (DOHH-2, K562 and Jobo-1). The cells were fixed and analyzed for VP3 expression by staining with monoclonal CVI-CAV-85.1 and induction of apoptosis by staining with propidium iodide. Early after transfection, VP3 positive cells were observed with a fine-granulate distribution of VP3 in the nucleus which was stained with propidium iodide and VP3 positive cells with nuclei containing VP3 aggregates with nuclei that did not stain with propidium iodide. The percentage of VP3 positive cells with nuclei that did not stain with propidium iodide and contained VP3 aggregates was found for the 4 different hematopoietic cell lines to range between 75 and 95%, 5 days after transfection (FIG. **11**A). Then K562 cells were transfected with DNA of the plasmid pCMV-tr which expresses C terminal truncated VP3. Expression of truncated VP3 in K562 cells induced cell death much less efficient than wild type VP3.

Our conclusion is that expression of VP3 in human hematopoietic tumor cells leads to specific induction of apoptosis. Expression of VP3 in the human breast tumor cell line MCF-7 (Lippmann, et al., 1980) also resulted in the induction of apoptosis (Noteborn, et al., results not published).

In the literature it is described that (human) tumors and tumor cell lines that do not contain functional p53 are less/not susceptible to induction of cell death by chemotherapeutics and radiation treatment (Lower et al., 1993). The tumor suppressor gene p53 acts as intermediary in the induction of apoptosis by specific anti-tumor agents. We have examined whether VP3 is capable of inducing apoptosis in human cells that do not possess p53 or possess mutated p53. VP3 was expressed in human osteosarcoma cells by means of DEAE-dextran transfection with plasmid pCMV-VP3. The osteosarcoma-derived Saos-2 cells cannot synthesize p53, and Saos-2/Ala143 cells express mutated and thus non-function p53. As a positive control the U2-OS cell line containing wild type p53 was used (Diller et al., 1990). The results given in FIG. **12**A show that VP3 can induce apoptosis in a comparable degree in cells that are $p53^-$ (p53 minus) (Saos-2 and Saos-2/Ala143) or $p53^+$ (U2-OS). Six days after transfection most of the VP3 positive cells are apoptotic. Expression of truncated VP3 induced much less efficient apoptosis in Saos-2 cells (FIG. **12**B). Our conclusion is the VP3 can specifically induce apoptosis inhuman tumor cells containing or not containing the tumor suppressor gene p53.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now having been fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 30

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GATCCAACCC GGGTTG                                                     16
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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AATTCAACCC GGGTTG                                                     16
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 449 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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Met Ala Arg Arg Ala Arg Arg Pro Arg Gly Arg Phe Tyr Ser Phe Arg
  1               5                  10                  15

Arg Gly Arg Trp His His Leu Lys Arg Leu Arg Arg Arg Tyr Lys Phe
             20                  25                  30

Arg His Arg Arg Arg Gln Arg Tyr Arg Arg Arg Ala Phe Arg Lys Ala
         35                  40                  45

Phe His Asn Pro Arg Pro Gly Thr Tyr Ser Val Arg Leu Pro Asn Pro
     50                  55                  60

Gln Ser Thr Met Thr Ile Arg Phe Gln Gly Val Ile Phe Leu Thr Glu
 65                  70                  75                  80

Gly Leu Ile Leu Pro Lys Asn Ser Thr Ala Gly Gly Tyr Ala Asp His
                 85                  90                  95

Met Tyr Gly Ala Arg Val Ala Lys Ile Ser Val Asn Leu Lys Glu Phe
            100                 105                 110

Leu Leu Ala Ser Met Asn Leu Thr Tyr Val Ser Lys Ile Gly Gly Pro
        115                 120                 125
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-continued

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Thr Ala Gly Glu Leu Ile Ala Asp Gly Ser Lys Ser Gln Ala Ala Asp
    130                 135                 140

Asn Trp Pro Asn Cys Trp Leu Pro Leu Asp Asn Asn Val Pro Ser Ala
145                 150                 155                 160

Thr Pro Ser Ala Trp Trp Arg Trp Ala Leu Met Met Met Gln Pro Thr
                165                 170                 175

Asp Ser Cys Arg Phe Phe Asn His Pro Lys Gln Met Thr Leu Gln Asp
            180                 185                 190

Met Gly Arg Met Phe Gly Gly Trp His Leu Phe Arg His Ile Glu Thr
        195                 200                 205

Arg Phe Gln Leu Leu Ala Thr Lys Asn Glu Gly Ser Phe Ser Pro Val
    210                 215                 220

Ala Ser Leu Leu Ser Gln Gly Glu Tyr Leu Thr Arg Arg Asp Asp Val
225                 230                 235                 240

Lys Tyr Ser Ser Asp His Gln Asn Arg Trp Gln Lys Gly Gly Gln Pro
                245                 250                 255

Met Thr Gly Gly Ile Ala Tyr Ala Thr Gly Lys Met Arg Pro Asp Glu
            260                 265                 270

Gln Gln Tyr Pro Ala Met Pro Pro Asp Pro Pro Ile Ile Thr Ala Thr
        275                 280                 285

Thr Ala Gln Gly Thr Gln Val Arg Cys Met Asn Ser Thr Gln Ala Trp
    290                 295                 300

Trp Ser Trp Asp Thr Tyr Met Ser Phe Ala Thr Leu Thr Ala Leu Gly
305                 310                 315                 320

Ala Gln Trp Ser Phe Pro Pro Gly Gln Arg Ser Val Ser Arg Arg Ser
                325                 330                 335

Phe Asn His His Lys Ala Arg Gly Ala Gly Asp Pro Lys Gly Gln Arg
            340                 345                 350

Trp His Thr Leu Val Pro Leu Gly Thr Glu Thr Ile Thr Asp Ser Tyr
        355                 360                 365

Met Ser Ala Pro Ala Ser Glu Leu Asp Thr Asn Phe Phe Thr Leu Tyr
    370                 375                 380

Val Ala Gln Gly Thr Asn Lys Ser Gln Gln Tyr Lys Phe Gly Thr Ala
385                 390                 395                 400

Thr Tyr Ala Leu Lys Glu Pro Val Met Lys Ser Asp Ala Trp Ala Val
                405                 410                 415

Val Arg Val Gln Ser Val Trp Gln Leu Gly Asn Arg Gln Arg Pro Tyr
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Pro Asn Asp Val Asn Trp Ala Asn Ser Thr Met Tyr Trp Gly Thr Gln
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Pro
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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1348 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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CCACCTCAAG CGACTTCGAC GAAGATATAA ATTTCGACAT CGGAGGAGAC AGCGGTATCG    120

TAGACGAGCT TTTAGGAAGG CCTTTCACAA CCCCCGCCCC GGTACGTATA GTGTGAGGCT    180

GCCGAACCCC CAATCTACTA TGACTATCCG CTTCCAAGGG GTCATCTTTC TCACGGAAGG    240

ACTCATTCTG CCTAAAAACA GCACAGCGGG GGGCTATGCA GACCACATGT ACGGGGCGAG    300

AGTCGCCAAG ATCTCTGTGA ACCTGAAAGA GTTCCTGCTA GCCTCAATGA ACCTGACATA    360

CGTGAGCAAA ATCGGAGGCC CCATCGCCGG TGAGTTGATT GCGGACGGGT CTAAATCACA    420

AGCCGCGGAC AATTGGCCTA ATTGCTGGCT GCCGCTAGAT AATAACGTGC CCTCCGCTAC    480

ACCATCGGCA TGGTGGAGAT GGGCCTTAAT GATGATGCAG CCCACGGACT CTTGCCGGTT    540

CTTTAATCAC CCAAAGCAGA TGACCCTGCA AGACATGGGT CGCATGTTTG GGGGCTGGCA    600

CCTGTTCCGA CACATTGAAA CCCGCTTTCA GCTCCTTGCC ACTAAGAATG AGGGATCCTT    660

CAGCCCCGTG GCGAGTCTTC TCTCCCAGGG AGAGTACCTC ACGCGTCGCG ACGATGTTAA    720

GTACAGCAGC GATCACCAGA ACCGGTGGCA AAAAGGCGGA CAACCGATGA CGGGGGGCAT    780

TGCTTATGCG ACCGGGAAAA TGAGACCCGA CGAGCAACAG TACCCTGCTA TGCCCCCAGA    840

CCCCCCGATC ATCACCGCTA CTACAGCGCA AGGCACGCAA GTCCGCTGCA TGAATAGCAC    900

GCAAGCTTGG TGGTCATGGG ACACATATAT GAGCTTTGCA ACACTCACAG CACTCGGTGC    960

ACAATGGTCT TTTCCTCCAG GGCAACGTTC AGTTTCTAGA CGGTCCTTCA ACCACCACAA   1020

GGCGAGAGGA GCCGGGGACC CCAAGGGCCA GAGATGGCAC ACGCTGGTGC CGCTCGGCAC   1080

GGAGACCATC ACCGACAGCT ACATGTCAGC ACCCGCATCA GAGCTGGACA CTAATTTCTT   1140

TACGCTTTAC GTAGCGCAAG GCACAAATAA GTCGCAACAG TACAAGTTCG GCACAGCTAC   1200

ATACGCGCTA AAGGAGCCGG TAATGAAGAG CGATGCATGG GCAGTGGTAC GCGTCCAGTC   1260

GGTCTGGCAG CTGGGTAACA GGCAGAGGCC ATACCCATGG GACGTCAACT GGGCGAACAG   1320

CACCATGTAC TGGGGGACGC AGCCCTGA                                      1348
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 216 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
Met His Gly Asn Gly Gly Gln Pro Ala Ala Gly Gly Ser Glu Ser Ala
  1               5                  10                  15

Leu Ser Arg Glu Gly Gln Pro Gly Pro Ser Gly Ala Ala Gln Gly Gln
             20                  25                  30

Tyr Ile Ser Asn Glu Arg Ser Pro Arg Arg Tyr Ser Thr Arg Thr Ile
         35                  40                  45

Asn Gly Val Gln Ala Thr Asn Lys Phe Thr Ala Val Gly Asn Pro Ser
     50                  55                  60

Leu Gln Arg Asp Pro Asp Trp Tyr Arg Trp Asn Tyr Asn His Ser Ile
 65                  70                  75                  80

Ala Val Trp Leu Arg Glu Cys Ser Arg Ser His Ala Lys Ile Cys Asn
                 85                  90                  95

Cys Gly Gln Phe Arg Lys His Asn Phe Gln Glu Cys Ala Gly Leu Glu
            100                 105                 110
```

-continued

```
Asp Arg Ser Thr Gln Ala Ser Leu Glu Glu Ala Ile Leu Arg Pro Leu
        115                 120                 125

Arg Val Gln Gly Lys Arg Ala Lys Arg Lys Leu Asp Tyr His Tyr Ser
    130                 135                 140

Gln Pro Thr Pro Asn Arg Lys Lys Ala Tyr Lys Thr Val Arg Trp Gln
145                 150                 155                 160

Asp Glu Leu Ala Asp Arg Glu Ala Asp Phe Thr Pro Ser Glu Glu Asp
                165                 170                 175

Gly Gly Thr Thr Ser Ser Asp Phe Asp Glu Asp Ile Asn Phe Asp Ile
            180                 185                 190

Gly Gly Asp Ser Gly Ile Val Asp Glu Leu Leu Gly Arg Pro Phe Thr
        195                 200                 205

Thr Pro Ala Pro Val Arg Ile Val
    210                 215
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 651 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
ATGCACGGGA ACGGCGGACA ACCGGCCGCT GGGGGCAGTG AATCGGCGCT TAGCCGAGAG     60

GGGCAACCTG GGCCCAGCGG AGCCGCGCAG GGGCAAGTAA TTTCAAATGA ACGCTCTCCA    120

AGAAGATACT CCACCCGGAC CATCAACGGT GTTCAGGCCA CCAACAAGTT CACGGCCGTT    180

GGAAACCCCT CACTGCAGAG AGATCCGGAT TGGTATCGCT GGAATTACAA TCACTCTATC    240

GCTGTGTGGC TGCCCGAATG CTCGCGCTCC CACGCTAAGA TCTGCAACTG CGGACAATTC    300

AGAAAGCACT GGTTTCAAGA ATGTGCCGGA CTTGAGGACC GATCAACCCA AGCCTCCCTC    360

GAAGAAGCGA TCCTGCGACC CCTCCGAGTA CAGGGTAAGC GAGCTAAAAG AAAGCTTGAT    420

TACCACTACT CCCAGCCGAC CCCGAACCGC AAAAAGGCGT ATAAGACTGT AAGATGGCAA    480

GACGAGCTCG CAGACCGAGA GGCCGATTTT ACTCCTTCAG AAGAGGACGG TGGCACCACC    540

TCAAGCGACT TCGACGAAGA TATAAATTTC GACATCGGAG GAGACAGCGG TATCGTAGAC    600

GAGCTTTTAG GAAGGCCTTT CACAACCCCC GCCCCGGTAC GTATAGTGTG A             651
```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 121 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
Met Asn Ala Leu Gln Glu Asp Thr Pro Pro Gly Pro Ser Thr Val Phe
  1               5                  10                  15

Arg Pro Pro Thr Ser Ser Arg Pro Leu Glu Thr Pro His Cys Arg Glu
             20                  25                  30

Ile Arg Ile Gly Ile Ala Gly Ile Thr Ile Thr Leu Ser Leu Cys Gly
```

-continued

```
        35                  40                  45

Cys Ala Asn Ala Arg Ala Pro Thr Leu Arg Ser Ala Thr Ala Asp Asn
    50                  55                  60

Ser Glu Ser Thr Gly Phe Lys Asn Val Pro Asp Leu Arg Thr Asp Gln
65                  70                  75                  80

Pro Lys Pro Pro Ser Lys Lys Arg Ser Cys Asp Pro Ser Glu Tyr Arg
                85                  90                  95

Val Ser Glu Leu Lys Glu Ser Leu Ile Thr Thr Thr Pro Ser Arg Pro
            100                 105                 110

Arg Thr Ala Lys Arg Arg Ile Arg Leu
        115                 120
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 366 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
ATGAACGCTC TCCAAGAAGA TACTCCACCC GGACCATCAA CGGTGTTCAG GCCACCAACA      60

AGTTCACGGC CGTTGGAAAC CCCTCACTGC AGAGAGATCC GGATTGGTAT CGCTGGAATT     120

ACAATCACTC TATCGCTGTG TGGCTGCGCG AATGCTCGCG CTCCCACGCT AAGATCTGCA     180

ACTGCGGACA ATTCAGAAAG CACTGGTTTC AAGAATGTGC CGGACTTGAG GACCGATCAA     240

CCCAAGCCTC CCTCGAAGAA GCGATCCTGC GACCCCTCCG AGTACAGGGT AAGCGAGCTA     300

AAAGAAAGCT TGATTACCAC TACTCCCAGC CGACCCCGAA CCGCAAAAAG GCGTATAAGA     360

CTGTAA                                                                366
```

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

```
Glu Asp Thr Pro Pro Gly Pro Ser Thr Val Phe Arg
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

```
Asp Thr Pro Pro Gly Pro Ser Thr Val Phe Arg Pro
1               5                   10
```

```
(2) INFORMATION FOR SEQ ID NO: 11:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 12 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Thr Pro Pro Gly Pro Ser Thr Val Phe Arg Pro Pro
1                 5                   10


(2) INFORMATION FOR SEQ ID NO: 12:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 12 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Pro Pro Gly Pro Ser Thr Val Phe Arg Pro Pro Thr
  1               5                   10


(2) INFORMATION FOR SEQ ID NO: 13:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 12 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Pro Gly Pro Ser Thr Val Phe Arg Pro Pro Thr Ser
  1               5                   10


(2) INFORMATION FOR SEQ ID NO: 14:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 12 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Gly Pro Ser Thr Val Phe Arg Pro Pro Thr Ser Ser
  1               5                   10


(2) INFORMATION FOR SEQ ID NO: 15:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 12 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: peptide
```

-continued

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

```
Pro Ser Thr Val Phe Arg Pro Pro Thr Ser Ser Arg
  1               5                  10
```

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

```
Gln Glu Cys Ala Gly Leu Glu Asp Arg Ser Thr Gln
  1               5                  10
```

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

```
Glu Cys Ala Gly Leu Glu Asp Arg Ser Thr Gln Ala
  1               5                  10
```

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

```
Cys Ala Gly Leu Glu Asp Arg Ser Thr Gln Ala Ser
  1               5                  10
```

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

```
Ala Gly Leu Glu Asp Arg Ser Thr Gln Ala Ser Leu
  1               5                  10
```

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

```
Gly Leu Glu Asp Arg Ser Thr Gln Ala Ser Leu Glu
  1               5                  10
```

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

```
Pro Ser Thr Val Phe Arg Pro Pro Thr Ser Ser Arg
  1               5                  10
```

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

```
Ser Thr Val Phe Arg Pro Pro Thr Ser Ser Arg Pro
  1               5                  10
```

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

```
Thr Val Phe Arg Pro Pro Thr Ser Ser Arg Pro Leu
  1               5                  10
```

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

```
Val Phe Arg Pro Pro Thr Ser Ser Arg Pro Leu Glu
```

-continued

```
 1               5                  10


(2) INFORMATION FOR SEQ ID NO: 25:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 12 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Phe Arg Pro Pro Thr Ser Ser Arg Pro Leu Glu Thr
 1               5                  10


(2) INFORMATION FOR SEQ ID NO: 26:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 12 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Arg Pro Pro Thr Ser Ser Arg Pro Leu Glu Thr Pro
 1               5                  10


(2) INFORMATION FOR SEQ ID NO: 27:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 12 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Pro Pro Thr Ser Ser Arg Pro Leu Glu Thr Pro His
 1               5                  10


(2) INFORMATION FOR SEQ ID NO:28:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 6 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: unknown
          (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Pro Ser Thr Val Phe Arg
1               5


(2) INFORMATION FOR SEQ ID NO:29:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 8 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: unknown
          (D) TOPOLOGY: unknown
```

-continued

```
    (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Gly Leu Glu Asp Arg Ser Thr Gln
1               5


(2) INFORMATION FOR SEQ ID NO:30:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 5 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: unknown
          (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Pro Thr Ser Ser Arg
1               5
```

What is claimed is:

**1**. A composition comprising:

a polypeptide derived from a Chicken Anemia Virus, free from its natural environment, which polypeptide comprises an amino acid sequence depicted in FIG. **3** (SEQ ID NO: 7).

**2**. A composition comprising:

a polypeptide derived from a Chicken Anemia Virus, free from its natural environment which polypeptide comprises an amino acid sequence depicted in FIG. **2** (SEQ ID NO: 5).

**3**. A composition said composition comprising:

a polypeptide derived from a Chicken Anemia Virus, free from its natural environment, which polypeptide comprises an amino acid sequence depicted in FIG. **3** (SEQ ID NO: 7), and further comprises a polypeptide comprising an amino acid sequence depicted in FIG. **2** (SEQ ID NO: 5).

**4**. An isolated polypeptide derived from a Chicken Anemia Virus, which polypeptide comprises the amino acid sequence depicted in SEQ ID NO: 5.

**5**. An isolated polypeptide derived from a Chicken Anemia Virus, which polypeptide comprises the amino acid sequence depicted in SEQ ID NO: 7.

**6**. A conjugate comprising:

one or both of a polypeptide according to claim **4** or claim **5** and a substance which has affinity for tumor cell associated proteins, sugars or lipids.

**7**. The conjugate according to claim **6**, wherein said substance which has affinity for tumor cell associated proteins, sugars or lipids is an antibody, a derivative of an antibody or a fragment of an antibody.

**8**. The conjugate according to claim **6**, wherein said substance that has affinity for tumor cell associated proteins, sugars or lipids, is liposome encapsulated.

**9**. A method for inhibiting growth of a tumor, said method comprising:

contacting said tumor with a sufficient amount of a composition according to claim **1** or claim **3** or an isolated polypeptide according to claim **4** or **5** to inhibit growth of said tumor.

**10**. The method according to claim **9**, wherein said tumor is a solid tumor and said providing is physically introducing said composition into said tumor.

**11**. The method according to claim **10**, wherein said physically introducing is injecting said composition directly into said tumor.

**12**. A method for inducing apoptosis in a tumor cell, said method comprising:

producing in said tumor cell a sufficient amount of a composition comprising:

one or both of a polypeptide having the sequence as depicted in FIG. **3** (SEQ ID NO: 7) or FIG. **2** (SEQ ID NO: 5), so that apoptosis is induced in said tumor cell.

**13**. The method according to claim **12**, wherein said tumor cell is a mammalian tumor cell.

**14**. The method according to claim **13**, wherein said mammalian tumor cell is a human tumor cell.

**15**. A method for inducing tumor cell death, said method comprising:

producing in said tumor cell a sufficient amount of a composition comprising one or both of a polypeptide having the sequence as depicted in FIG. **3** (SEQ ID NO: 7) or FIG. **2** (SEQ ID NO: 5), to induce death in said tumor cell.

**16**. A method for inhibiting growth of a tumor cell, said method comprising:

producing in a tumor cell a sufficient amount of a composition comprising one or both of a polypeptide having the sequence as depicted in FIG. **3** (SEQ ID NO: 7) or FIG. **2** (SEQ ID NO: 5), whereby growth of said tumor cell is inhibited.

**17**. The method according to claim **16**, wherein said cell is in vivo.

**18**. A method for inducing tumor cell death, said method comprising:

transfecting said tumor cell with an expression vector comprising a nucleotide sequence encoding one or both of a polypeptide as depicted in FIG. **3** (SEQ ID NO: 7) or FIG. **2** (SEQ ID NO: 5), whereby said polypeptide is expressed in said tumor cell, thereby inducing tumor cell death.

**19**. A method for inhibiting growth of a tumor cell, said method comprising:

transfecting said tumor cell with an expression vector comprising a nucleotide sequence encoding one or both of a polypeptide as depicted in FIG. **3** (SEQ ID NO: 7) or FIG. **2** (SEQ ID NO: 5), whereby said polypeptide is expressed in said tumor cell.

**20**. A method for inducing apoptosis in a tumor cell, said method comprising:

transfecting said cell with an expression vector comprising a nucleotide sequence encoding one or both of a polypeptide as depicted in FIG. **3** (SEQ ID NO: 7) or FIG. **2** (SEQ ID NO: 5), whereby said polypeptide is expressed in said tumor cell, thereby inducing apoptosis.

**21**. The method according to any one of claims **15** and **18–20**, wherein said cell is in vivo.

**22**. A method for reducing an autoimmune cell population, said method comprising:

endogenously expressing in a mammalian host one of both of a polypeptide depicted in FIG. **3** (SEQ ID NO: 7) or FIG. **2** (SEQ ID NO: 5) so that apoptosis is induced and said autoimmune cell population is reduced.

**23**. A method of reducing a transformed cell population, said method comprising:

endogenously expressing in a mammalian host one or both of a polypeptide depicted in FIG. **3** (SEQ ID NO: 7) or FIG. **2** (SEQ ID NO: 5), whereby said transformed cell population is reduced.

**24**. The method according to claim **23**, wherein said transformed cell population is a cancer cell population.

**25**. A method for inducing apoptosis in tumor cells, said method comprising:

introducing into said tumor cells a DNA molecule coding for one or both polypeptides depicted in FIG. **2** (SEQ ID NO: 5) or FIG. **3** (SEQ ID NO: 7), under conditions whereby said cells express said DNA, whereby apoptosis in said tumor cells is induced.

**26**. The method according to claim **25**, wherein said introducing of said DNA into said tumor cells is via a viral vector.

**27**. The method according to claim **25**, wherein said introducing of said DNA into said tumor cells is via receptor-mediated uptake.

**28**. The method according to claim **25**, wherein said introducing of said DNA into said tumor cells is via liposomes.

**29**. The method according to claim **25**, wherein said introducing of said DNA into said tumor cells is via direct injection into a tumor comprising said tumor cells.

**30**. The method according to claim **25**, wherein said introducing of said DNA into said tumor cells is via particle bombardment.

**31**. A method for inducing tumor cell death, said method comprising:

introducing into said tumor cells a DNA molecule coding for one or both polypeptides depicted in FIG. **2** (SEQ ID NO: 5) or FIG. **3** (SEQ ID NO: 7), under conditions whereby said cells express said DNA, whereby death in said tumor cells is induced.

**32**. The method according to claim **31**, wherein said introducing of said DNA into said tumor cells is via a viral vector.

**33**. The method according to claim **31**, wherein said introducing of said DNA into said tumor cells is via receptor-mediated uptake.

**34**. The method according to claim **31**, wherein said introducing of said DNA into said tumor cells is via liposomes.

**35**. The method according to claim **31**, wherein said introducing of said DNA into said tumor cells is via direct injection into a tumor comprising said tumor cells.

**36**. The method according to claim **31**, wherein said introducing of said DNA into said tumor cells is via particle bombardment.

**37**. A method for inhibiting tumor growth, said method comprising:

introducing into tumor cells a DNA molecule coding for one or both polypeptides depicted in FIG. **2** (SEQ ID NO: 5) or FIG. **3** (SEQ ID NO: 7), under conditions whereby said cells express said DNA, whereby growth of said tumor comprising of said tumor cells is inhibited.

**38**. The method according to claim **37**, wherein said introducing of said DNA into said tumor cells is via a viral vector.

**39**. The method according to claim **37**, wherein said introducing of said DNA into said tumor cells is via receptor-mediated uptake.

**40**. The method according to claim **37**, wherein said introducing of said DNA into said tumor cells is via liposomes.

**41**. The method according to claim **37**, wherein said introducing of said DNA into said tumor cells is via direct injection into a tumor comprising said tumor cells.

**42**. The method according to claim **37**, wherein said introducing of said DNA into said tumor cells is via particle bombardment.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

# CERTIFICATE OF CORRECTION

PATENT NO. : 6,162,461



DATED : December 19, 2000

INVENTOR(S) : M. Noteborn, G. Koch

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 3,
Line 51, before "pA=polyadenylation", add -- TAA=stop codon --.

Column 4,
Line 42, "-□—Sa05-2/'" should read -- -□-=Saos-2/ --.
Line 43, "-O-=Sa05-2, p53-" should read -- -O-=Saos-2, p53- -- and

**"-●-=U2-05, p53t" should read -- -●-=U2-OS, p53+ --.**

Line 44, "-O-=Sa05-2*pCMV-VP3" should read -- -O-=Saos-2*pCMV-VP3 -- and

**"-●-=Sa05-" should read -- -●-=Saos- --.**

Column 7,
Line 12, "pace" should read -- place --.
Line 18, "pails" should read -- parts -- .

Claim 9,
Line 66, "according to claim 4 or 5" should read -- derived from a Checken Anemia Virus, which polypeptide comprises the amino acid sequence depicted inSEQ ID NO: 5, or the amino acid sequence depicted in SEQ ID NO:7, --

Claim 10,
Line 28, delete "said providing in physically introducing".
Line 29, after "composition", insert -- is physically introduced --.

Signed and Sealed this

Eighth Day of January, 2002

*Attest:*

JAMES E. ROGAN

*Attesting Officer*

*Director of the United States Patent and Trademark Office*